

France's blood scandal draws blood

The trial in France of four government officials for complicity in the use of contaminated blood may have ended with the verdicts announced last Friday, but the recriminations will continue.

NOT often are scientists in the public service sent to jail, but that is what may happen as a consequence of last week's verdicts by a Paris court (see page 764). At issue is the criminal case against four former government officials, two from the French Central Blood Transfusion Service (CNTS). The charges stem from the delay, between March and October 1985, in the use of heat-shock treatment of transfusion blood to rid it of contamination by the virus now known as HIV. One result is that hundreds of people, notably haemophiliacs dependent on Factor VIII, were infected with AIDS. Some of them have died. And France has been up in arms since it became plain, earlier this year, that the delay was a scandal of public administration.

Sadly, the court's long judgement (nearly 100 pages of it) throws little light on the reasons for what went wrong. One drawback is the nature of the charges on which last week's sentences are based. Technically, the convictions are for fraud in the sense in which it applies in the field of consumer protection; those convicted are held to have "sold, or allowed to be sold", products that were not what their eventual consumers had a right to expect. If it had been otherwise, the sentences could well have been more severe; four years, the sentence meted out to Dr Michel Garretta, the previous director of the CNTS, is the maximum allowed.

So are French haemophiliacs and their relatives right in their complaint that criminals have now escaped lightly? At this remove, it is not easy to reconstruct the circumstances of early 1985. At the beginning of the year, the feasibility of a blood test for HIV had been demonstrated just seven months earlier. A number of companies in the United States had immediately embarked on the development of a test, which came into use later in 1984, but whose limitations were still being defined in the early months of 1985. Manufacturers elsewhere, in France included, were further behind. Yet the incidence of AIDS among haemophiliac patients had already been recognized; the inevitable inference was that they had been given Factor VIII from contaminated blood. From that point (the end of 1984), it was indefensible that untested untreated blood should be given to anybody.

That is what the French court has decided. The most damaging evidence is that those concerned knew what the risks were, but nevertheless persisted with the use of untreated blood "while stocks lasted". Why? Or rather, why on Earth? There are two explanations: the cost of throwing away a huge stock of transfusion blood would have been considerable, while Garretta was persuaded of what the

court calls the "industrial logic" of waiting until the required processes and materials were available in France. It is not a creditable story. France is rightly angry.

Whether the sentences are just is another matter. Although CNTS is a government dependency, its policies and practices must be approved by its sponsoring ministry in the light of the usual advice from advisory committees. The court last week pinned chief responsibility on Garretta, saying that he did nothing to alert his superiors to the dangers. But should not others have pointed out the risks? As it happens, they did so. Professor Jacques Roux, director general of health in 1985, who had been warned, was given a suspended sentence of four years for having failed to challenge Garretta's policy and for having failed to warn his political masters with sufficient urgency of the dangers. Professor Jean-Pierre Allain, now a professor at the University of Cambridge but then a subordinate of Garretta, produced in his defence a letter written to his chief in January 1985 demanding a decision by the end of the month on a planned contract to build a heat-treatment plant. In the court's view, Allain's fault is to have kept quiet after his demand had been refused, and in particular to have failed to warn l'Association Française des Hémophiles, at a meeting in May, of what was afoot. Allain is sentenced to four years in jail, with two suspended.

There is a disconcerting element of populism in these decisions. Nobody denies that the continued use of contaminated blood for the best part of 1985 was a scandal. Those unwittingly infected with HIV are proof that the consequences of bureaucratic bungling can be tragic as well as costly. But the key decisions were bureaucratic decisions. The officials prosecuted on an inappropriate charge appear to have been selected at random. And nothing has been learned of whether technical decisions were compromised by political considerations. The best course now would be that the government should set up a formal judicial enquiry to discover how this disastrous decision came to be made. Meanwhile, public servants everywhere will have been given a fright. □

Tainted academics

Eastern Germany is making needless heavy weather over the status of academics.

THE two great symbolic events in recent European history are the construction of the Berlin Wall in 1961, which



seemed to signify that the Cold War had come to stay, and its collapse almost three decades later, which seemed to mark the beginning of a period of sweetness and light for Central Europe. In reality, both expectations have been falsified. The Cold War has gone. And reunited Germany is struggling through an unaccustomed spell of economic difficulty brought on by the need to borrow funds to set the five new eastern *Länder* on their feet. Now there are people throughout Germany who believe they are worse off than before. The recent riots against the presence of immigrants in Rostock, and other eastern German towns, is one measure of the discontent occasioned by the wall's collapse.

Another, in its way more serious because its shadow is certain to be long, is the trouble in eastern German universities over the status of academics who, in the shameful past, have enjoyed the favours of the ruling Communist party. It is a haunting problem. To tell that, put yourself in the shoes of a young scientist in one of the Stalinist states of Central Europe in the early 1960s. (Stalin was dead, but Khrushchev's reforms did not touch the Central Committee's control of how the satellite states behaved.) An able person then would have known that, to succeed, he or she would have had to make intellectual bridges with people elsewhere, preferably in the West. But, naturally, exit visas would have been awarded more readily to members of the Party. Moreover, people travelling overseas on business trips would have been required, on their return, to make their peace with the local secret police, the notorious *Stasi* in East Germany. How else could a young person hope to succeed?

The result has nevertheless been an embarrassment throughout Central Europe since the Berlin Wall came down. In Poland, eastern Germany, Czechoslovakia and Hungary, the scientists best-known in the West are those who appeared at international conferences (often, desperately, at the last minute) under Party patronage. Their less compliant colleagues, required to make do instead with collaborations in the Soviet Union, are by comparison obscure. How should that army of the disadvantaged respond to the continued presence in academic life of those who took advantage of the Party's past munificence, often acquiring power over their fellows in the process? It is a haunting issue, but one the universities of eastern Germany should be tackling in a more enlightened spirit than that outlined on page 762.

There must be a better way. Tainted researchers and academics, it appears, are being told by commissions that their appointments have been terminated, and are then required (if they have the stomach) to fight their cases through the courts. What should matter more is whether a person compromised by past conduct can yet command the respect of colleagues. Much must hang on colleagues' estimation of compromised people's value as scientists, but also on the terms in which they have sought to explain to colleagues that their previous connections with now-defunct regimes were innocent of malign intent. Indeed, such collegial determinations, whether by universities or research institutes, of a person's continued tenure of his post, are alone able to carry weight. It is a cruel corollary that there can

be no way of making up for past injustice, say the unjust denial of promotion or of exit visas to able people for decades on end. That, sadly, is water under the bridge of history.

The danger in the present way of handling the status of academics compromised by their past is that it will needlessly prolong the inevitable sense of bitterness in German academic institutions at the injustices of the past. Denial of due process now is a means of repaying past with present injustice. Since the Second World War, Germany has had a shining reputation for its scrupulous respect for constitutional procedures, even to the extent that failed medical students have been able to carry their discontents to the constitutional court at Karlsruhe. A more seemly way of defining the status of eastern German academics and researchers is in everybody's interest, the impending trial of Erich Hönicker notwithstanding. Academic institutions should take the lead. □

Diet and breast cancer

Studies show no correlation between dietary fat and breast cancer. It is time to move on.

If ever proof were needed that the proposition that there is a cause-and-effect relationship between diet and breast cancer far exceeds scientific data, the US National Institutes of Health's plan to conduct a \$10 million clinical trial is proof indeed. Despite abundant evidence that dietary fat bears no relation to development of cancer of the breast, the NIH intends (under the fashionable umbrella of "women's health") to initiate a study of 40,000 women (half of whom will be randomly assigned to consume no more than 20 per cent of their calories in fat) to try once again to prove a link that is probably not there.

Last week, a team of researchers from the Harvard-affiliated Brigham and Women's Hospital in Boston reported in the 21 October issue of the *Journal of the American Medical Association* (240, 2037-2044; 1992) that they found no correlation between dietary fat and breast cancer in a continuing eight-year study of 89,494 women between the ages of 34 and 59. This latest study is the largest but by no means the only one to reach this conclusion, which is a great disappointment to those who wish to discover that breast cancer is something over which we have control. But another study of 35,000 women, as well as one including more than 5,000 women, reached the same unpopular conclusion. (Data from the Brigham study do confirm a correlation between fat intake and colon cancer, so that hamburgers and french fries can still be fairly pilloried.)

Why then does NIH insist on spending \$10 million on a study whose hypothesis seems to be little more than wishful thinking? Is it only because of the faddish infatuation with fat as the root of all dietary evil? In the United States, as elsewhere, money for scientific research is in short supply. There are many ways the NIH could better spend its \$10 million. It is not too late to face the facts, however disappointing. □

Rumblings grow about Parkfield in wake of first earthquake prediction

Pasadena. Seismologists and California officials last week pronounced the first official short-term earthquake prediction a success despite the fact that the event did not occur. But the activity has renewed criticism of a \$19-million project in a remote region of central California.

The prediction, triggered by a magnitude 4.7 earthquake last week in Parkfield (population 34), warned of a 37 per cent chance of a magnitude 6 earthquake for a 72-hour period beginning on Monday, 19 October. This past Sunday evening, after the Level A alert was withdrawn, a Level B alert was issued when tremors of magnitude 3.4 and 3.9 struck the area. That means a 10 per cent chance of a magnitude 6 earthquake in the next 72 hours.

Parkfield, located on the San Andreas fault midway between Los Angeles and San Francisco, is the subject of intense study because of a remarkably regular series of magnitude 6 earthquakes every 22 years or so beginning in 1857. In 1985, the United States Geological Survey (USGS) launched the Parkfield Prediction Experiment after declaring that there was a 95 per cent chance of another magnitude 6 earthquake by January 1993.

The project has saturated the area around Parkfield with equipment to measure the earthquake and a variety of possible precursors, including changes in the Earth's magnetic field and in the water levels of local wells. But the expected earthquake has so far failed to occur, and the controversial experiment is criticized for having a serious statistical flaw and for diverting money and resources that might be better used in areas likely to be struck by far larger and more damaging earthquakes.

The statistical criticism comes from Mark Matthews, a statistician at the Massachusetts Institute of Technology, who says that the 1985 forecast was made by omitting an event in 1934, which inconveniently occurred only 12 years after the previous earthquake and 32 years before the next one. Although the authors of the 1985 study maintained that they had valid physical reasons for believing that the 1934 earthquake was uncharacteristic, Matthews maintains that these justifications are "simplistic and wishful" and "not really scientifically defensible".

Matthews' own calculations, which include the 1934 earthquake, indicate that the next event after 1966 would have been expected in 1982, ± 16.6 years. In addition, his calculations show that the probability of the magnitude 6 earthquake happening between 1983 and 1993 (the original forecast interval) is only 40 per cent.



There were more questions than answers when scientists like John Langbein of the US Geological Survey met the media at Parkfield.

USGS geophysicist Andy Michael agrees that the experiment may have been "oversold" but argues that Parkfield still gives seismologists their best chance of observing a significant earthquake within a relatively short time. He says that the experiment should continue beyond January whether or not the predicted earthquake occurs. Michael says that a similar experiment elsewhere would be expensive and that seven years of accumulated baseline data at Parkfield is irreplaceable. An external review board is scheduled to issue a report in December on the future of the project, which costs

\$2 million a year to operate.

One problem with the experiment is that its short-term prediction was based on only a 37 per cent chance of an earthquake. Some worry that too many false alarms will cause the public to disregard future warnings. Michael, however, points out that short-term earthquake predictions with higher probabilities are as yet impossible. As a result, seismologists and state officials long ago decided to err on the side of providing the public with more rather than less information in the belief that the public would be outraged if seismologists failed to announce a prediction that proved correct.

Even critics agree that the experiment has improved communications between scientists and government planners. State officials regard even a failed earthquake prediction in a remote area as valuable preparation for larger earthquakes in both northern and southern California. In response to the Parkfield alert, for example, seven California counties within 100 miles of the epicentre activated their emergency-operations centres, and local police, fire and medical-services agencies took such steps as alerting personnel, reviewing emergency plans, making sure emergency generators were running and moving emergency vehicles out of doors.

The public also took precautions by buying more bottled water, flashlights and batteries. It seems that, to most Californians, the prediction was simply another reminder of the inevitability of a Big One.

Robert Finn

Groups sue to end fetal tissue ban

Washington. Following the latest failure to overturn the US ban on research using fetal tissue, five disease and research groups took matters into their own hands last week and sued the federal government.

The groups, which include the Association of American Medical Colleges, the Association of American Universities and three disease groups, claim that the US Department of Health and Human Services violated the Administrative Procedures Act when, in 1989, it made permanent a temporary ban on the research without following

the law on proper public notice. Congress has tried several times to overturn the ban but has failed to muster the two-thirds majority needed to overturn a presidential veto.

But events may overtake the lawsuit. Congress has promised to try to repeal the ban early next year and supporters believe that they have the votes to win. A Democrat in the White House would render the whole issue moot because overturning the ban is part of the party's platform.

Christopher Anderson

German professors appeal their dismissals in purge of thousands with ties to Stasi

Leipzig. Hundreds of university academics are appealing against their dismissals as part of the restructuring of the university system in the former East Germany. They have been judged by state commissions to be unfit for academic life because of allegations that they collaborated with the Stasi, East

Germany's secret service, or used their Communist Party connections to influence the careers of colleagues or students. But some of the dismissals appear instead to be a form of revenge by colleagues with personal grievances.



Armin Ermisch

The academics are pursuing their struggle individually, with no umbrella organization to protect their interests. But western scientists have lent their voices to defend Armin Ermisch, a professor of cell biology at the University of Leipzig, against reprisals for actions that, under normal circumstances, would have been considered perfectly appropriate.

Ermisch, whose international publication record in the past decade is well above average, has never hidden his early enthusiasm for communism, which he saw as the best possible option for post-war Germany. Neither does he hide his subsequent disillusion with the system or the fact that he chose to work from within to reform it. But he says that he never abused his status as a party member.

Ermisch has asked the state ministry to provide examples of actual wrongdoing, believing that committee members are reacting not to his politics but rather to the rigorously applied criteria of excellence which he enforced in his department. Those judged by Ermisch to be second-rate researchers were not promoted; unfortunately for him, these are now the people with power.

Immediately after reunification, Ermisch won an overwhelming vote of confidence in the university's first free and secret ballot. Yet only a year later he was kicked out as department chairman. "It was the worst day of my life", he says.

At a brief hearing in April, he was accused of being a member of the party and 'influencing the development of staff' in his section, 'influencing the education' of the students and providing information to Stasi 'on request'. In August he was given notice

of dismissal from the university by the state ministry of science and arts. Ermisch hopes that a court will hear his appeal before the end of this year, when his contract of employment expires. But a date next year is more likely, making remote the prospect of reinstatement.

Ermisch's cause is one of the few to reach the outside world. Foreign collaborators who have spent time in Leipzig have written open letters of support protesting about his treatment. John Russell, of Edinburgh University's physiology department, says that the commission's refusal to allow cross-examination of witnesses at the hearing is an assault on democracy, and he casts doubt on the motives of Ermisch's critics: "Old scores may very well be being settled here", he says.



Wolf-Dietrich Arnold

There are hundreds more in the same position. Wolf-Dietrich Arnold of the University of Leipzig's Orthopedic Clinic joined the Communist Party in 1985 because he believed it essential for a position he was offered as director of a large clinic in East Berlin. Ironically, he never took up the post, accepting instead a directorship created for him in Leipzig. Following a vote of confidence by his colleagues at the clinic, he worked normally and productively for two years. A hearing in July this year apparently went well, but his notification of dismissal in August came as a shock, and he was told to leave by the end of the year.

Arnold, one of 15 clinical professors at the clinic appealing against their removal for political reasons, has an impressive record of research on spinal injury, is known internationally and has received many job offers from other countries. He doesn't want to leave Germany and "live the life of a stranger", but he may have to if he cannot find work. He says that the official reasons for his dismissal are spurious and that a member of the commission that Arnold declined to promote has swung sentiment unfairly against him.

This belief is shared by at least one member of the commission, who has written in protest to the state research minister, Hans-Joachim Meyer. Arnold's lawyer, Friedel Schnur, representing all the clinical professors who were dismissed, says

that such a letter is not unusual.

Dismissals for reasons of 'personal integrity' are part of a broad package of staff redundancies and reorganizations in East Germany following reunification in November 1989. With universities chronically overstaffed, a sizeable number of dismissals were expected. Many professors were made redundant to save money, while others were found not to be qualified for their positions by evaluation committees made up of scientists from both eastern and western Germany.

Although the dismissals on grounds of incompetence caused disruption and unhappiness, they drew no strong charges of unfairness. The controversy surrounding those set up to judge political pasts reflects the more subjective nature of the investigations. Although the system is designed to be fair, in practice it is shadowy. And because exact figures are hard to come by, the extent of the problems is not known.

Responsibility for education is decentralized in Germany, and each of the five former east German states developed its own approach to restructuring under a directive outlined in the 1990 reunification contract. The general procedure involves

the establishment by the local science and education minister of small commissions to consider the 'personal integrity' of all professors and departmental chairpersons at a university, as well as others in positions of power. Known as 'Personal-



Hans-Ulrich Mönnig

kommissionen', these commissions have been conducting formal 'hearings' that, along with information from colleagues and from Stasi files, result in recommendations to the minister. The minister has sole responsibility for the final decision; the federal government is not involved.

The process attempts to be fair. The commissions usually include a reasonable cross-section of the academic community and the public. A commission will typically include two or three university professors, a lecturer and a student, along with a couple of professors from the west and, in many cases, relevant public figures. Some states allow universities to make the appointments, while others assign the task to the state minister.

But the lack of openness creates the opportunity for injustice, and although the decision can be appealed, a reversal in court does not necessarily guarantee reinstatement. A lengthy backlog of pending cases means that most of the vacated jobs will already have been filled. Even if reinstatement were offered, a lingering hostility may make working conditions intolerable. A more likely outcome of a successful appeal is monetary compensation.

In Sachsen, with the highest concentration of higher education institutes, about 1,000 academic staff have been dismissed for reasons of 'personal integrity'; nearly half have already left. Leipzig-based solicitor Karl Bönninger estimates that half of those dismissed are taking legal action, but so far no cases have been resolved in court, and in most cases the court hearing will be delayed for so long that the posts will be filled and the only recompense will be monetary.

In Sachsen-Anhalt, about 250, a quarter of the total redundancies, have been for political offences. Half are thought to be appealing their dismissal. Specific numbers in other states are not available, but in Thüringen around 250 people are likely to be involved, at least a third of whom may be seeking legal redress.

Although the restructuring of universities should have been completed within two years, it has been extended for a third year and may take even longer. To date, only one appeal has been completed, with the court finding in favour of the appellant.

Hans-Ulrich Mönnig, lodged a successful appeal against his dismissal as rector and professor at the University of Architecture and Civil Engineering in Weimar, Thüringen, although the state minister of research, Ulrich Fickel, fought unsuccessfully to overturn the ruling. Despite the experience, a result of a poor personal relationship between the two men, Mönnig believes that commissions are necessary to remove Stasi collaborators from public office and that the courts are capable of ensuring that justice prevails. At the same time, he thinks that the hearings and decisions should be open to public scrutiny and that the proceedings should be monitored by a qualified jurist.

Alison Abbott

Max Planck opens marine institute

Munich. The Max Planck Society, which funds science research institutes throughout Germany, this month opened its 57th variety — the Max Planck Institute for Marine Microbiology in Bremen. The new institute has two directors, and by 1996 plans to have a staff of 100, including 20 scientists. The research will focus on bacterial processes in marine environments.

Alison Abbott

Chinese scientist arrested for entrepreneurial spirit

Beijing. A Chinese scientist was arrested and thrown in jail for seven days by local authorities for his role in running a factory based on a product invented in his laboratory. The incident, which occurred in July, reflects the considerable hostility in parts of the country towards efforts by Communist Party leaders to support high-technology enterprises as a way of strengthening the nation's economy.

Zhang Shu-Lin, the 52-year-old director of the Jingzhou Kangbao New Technology Research Institute in Hubei Province in central China, visited Xiangfan City in Hubei to meet officials from the local high-technology development bureau planning a factory to manufacture a powerful detergent he had developed in his laboratory. Zhang had created a similar business in 1987 in cooperation with one of the city's neighbourhood committees, but Fandong government officials were jealous of the factory and after three years their opposition forced Zhang to quit as manager and bankrupted the company.

Since then, the State Science and Technology Commission has begun to encourage scientists to pursue commercially useful ideas, increasing government funding for such efforts. But its campaign has met with mixed success in much of the countryside outside the capital and selected enterprise zones in southern China. In those regions, the legacy of the 10-year Cultural Revolution, with its fervent anti-intellectualism, remains strong.

That attitude, combined with concern that the new factory posed unwanted com-

petition for local state-run enterprises, apparently led to Zhang's arrest on 3 July. Forcibly dragged from his temporary residence by Fandong District police, Zhang was handcuffed and paraded through the streets of the city amidst taunts of being "a stinking intellectual and an inventor".

While in prison, Zhang learned that his arrest resulted from an audit of his factory conducted two years earlier that had angered local authorities. Fortunately for Zhang, his situation was brought to the attention of the State Science and Technology Commission in Beijing, which managed to win his release on 10 July.

"This case is an example of what can happen when someone takes advantage of the increased opportunities for commercial activity in China and incurs the envy and anger of local officials", says Richard Dicker, a lawyer with Human Rights Watch in New York. "It offers a small but chilling insight into the many restrictions that exist on scientific activity in China."

The incident might have escaped notice had not the local party chief, Yang Bing-Qing, learned about Zhang's arrest a few weeks later. Yang promptly apologized to Zhang and promised to pursue those responsible. An investigation is continuing.

The Chinese press have decried the episode as demonstrating how much remains to be done to sell the government's message that the nation respects scientific talent and the fruits of its labour. Clearly, what is needed is an ongoing educational campaign about the value of knowledge.

You Qin Li

Scavengers undermine Indian incinerator

New Delhi. India's legions of rag-pickers have foiled the country's first waste incineration plant, causing it to close without having produced any electricity. The government is seeking \$10 million in damages from M/S Volund, the Danish contractor responsible for designing, building and operating the plant.

The waste plant, commissioned in 1988, was designed to generate 4 MW of electricity by burning 300 tonnes of waste daily. But neither the government nor the contractor took into consideration Delhi's 8,000 rag-pickers, who systematically retrieve reusable items such as wood, plastics, paper and cloth from municipal landfills. Unfortunately, once such combustible garbage is removed, the remains — mostly rotten vegetables and cooking wastes that do not easily burn — are not sufficient to operate the plant.

The Danish company was supposed to operate the plant for one year before handing it over to India's Department of Non-conventional Energy Sources (DNES), but engineers tried unsuccessfully for 18 months to get the plant working. DNES officials say that the company's engineers tested the quality of Delhi's garbage and found it adequate, and the government has asked for \$10 million to recover its investment.

Apart from the issue of the garbage, environmentalists have opposed the plant because it does not contain a flue-gas cleaning system to reduce levels of dioxin being emitted. The Danish company has been criticized for ignoring the fact that several municipal incinerators emitting high levels of dioxin were shut down in the Netherlands at the same time as the contract with India was being negotiated.

K.S. Jayaraman

Verdict in French blood trial shames science

Paris. Two former French blood transfusion officials, Michel Garretta and Jean-Pierre Allain, were given prison sentences last week for supplying haemophiliacs with clotting factors that they knew were infected with human immunodeficiency virus (HIV) and that could have been made safe by a heating process already in use in the United States and elsewhere. Although France was not the last European country to introduce heat-inactivated blood products, the court's ruling reinforces the fact that the risks of HIV infection through unheated products were known in 1985 when the decisions were made and were deliberately ignored for political and economic reasons.

The verdicts vindicate the persistent efforts of haemophiliac groups and shame the scientific community and health authorities, which failed to intervene despite adequate warning signs. At the same time, the trial did not address allegations that government officials delayed the approval of a US AIDS test for five months in 1985 so that a French test could be released first (see *Nature* 353, 197; 1991).

Garretta, former head of the National Blood Transfusion Centre, was sentenced to four years in prison and fined FF500,000 (US\$100,000), the maximum penalty, for "deception over the qualities of the blood products he sold". A warrant was issued for the arrest of Garretta, who is in Boston; his lawyers say he will return to face his sentence. Allain, former head of research at the

transfusion centre now working in Cambridge, England, was sentenced to four years in prison (two suspended) on the same charge.

The sentences affirm that the centre avoided importing heat-inactivated clotting



Garretta, shown during the trial, received a four-year prison sentence for allowing defective blood to be sold.

factors because France was waiting for the development of its own heat-activated products. Similarly, Garretta's decision to continue supplying contaminated products to haemophiliacs "until stocks are exhausted" seems to have been made on financial grounds.

While transfusion centre officials have been sentenced, the government has not been brought to account for its part in the

seven-month delay, which caused more than 1,500 haemophiliacs to be infected with HIV; more than 250 have since died. Although the government had been unequivocally informed by 21 March 1985 that its blood products were contaminated with HIV, the products were not withdrawn until 20 October. Two government officials charged with "non-assistance to persons in danger" received lenient treatment: Jacques Roux, former director general of the Health Ministry, was given a four-year suspended sentence, and Robert Netter, former head of the National Health Laboratory, was acquitted.

Ministers in the Socialist government at the time (including Laurent Fabius, then prime minister and now leader of the Socialist party) testified in court that their scientific advisers had not adequately informed them of the risks posed by the tainted blood products. How much the government knew, and when it knew it, may never be known, but what is clear is that effective safeguards were lacking, and that public confidence in the scientific community and health authorities has been shattered.

Although uncertainties existed in 1985 as to the seriousness of HIV infection, it now seems clear that there were enough warning signs to have expected the scientific and medical community to have intervened. However, only a few individuals, among them Jacques Liebowitch of the Raymond Poincaré Hospital outside Paris and François Pinon of the Cochin Hospital in Paris, tried to alert officials to the dangers of the blood products.

The public has interpreted this silence as complicity, a suspicion that has been heightened by the perceived reluctance of the government, the judiciary and the scientific community to take up the haemophiliacs' cause. The persistent efforts of haemophiliac groups to raise the issue of whether contaminated blood products had been deliberately distributed has now been vindicated, while the scientific community has been portrayed as too ready to defend the status quo. Moreover, concern that a suit against the French transfusion service would harm morale and set a dangerous precedent for other European countries (see *Nature* 353, 781; 1991) now appears to be misplaced.

But the blood transfusion saga is unlikely to end with the verdicts on Garretta and Allain. They intend to appeal, the haemophiliacs have started proceedings to obtain a more far-reaching retrial and opposition parties in the government have called for a trial of the Socialist politicians in power at the time.

Declan Butler

NIH and Army meet on breast cancer windfall

Washington. University researchers will benefit most from the \$210 million that the US Army has received for breast cancer research. The Army's decision to use the money for extramural, investigator-initiated projects approved through peer review by the National Institutes of Health (NIH) has reassured those concerned about its use but leaves unresolved NIH's role.

NIH and Army officials meeting last week to discuss the money awarded earlier this month in an amendment to the annual military appropriations bill (see *Nature* 359, 471; 1992) agreed to a task force of NIH researchers and Department of Defense (DOD) medical experts to suggest funding priorities. NIH officials are lobbying for basic research on such topics as potential environmental carcinogens and the role of chromosome 17, and would like at some point to include researchers and breast-cancer advocacy groups. The research

programme is expected to be set by February, with the money spent over two years.

The legislation ordered the military to cooperate with other federal agencies but did not specify to what degree. As a result, the Army is free either to hand over the entire project to NIH or to retain much of the responsibility for itself. It is not clear, for example, whether NIH will give the Army a list of projects to be funded and how much each should receive or whether it will simply submit a list of projects in order of scientific merit.

There is also concern that the Army will flood a few small areas with large amounts of money or select poorly from among competing fields of inquiry. For example, a project to develop digitized mammography machines is seen as an unwise use of some of the \$25 million it received last year to begin a breast cancer research programme.

Traci Watson

US panel rejects hiding source of allegations of misconduct

Washington. A US scientific advisory board has rejected proposed rules that would allow the government to keep secret files on a researcher, including testimony that could be used anonymously to support a finding of misconduct. At a meeting earlier this month, the Public Health Service (PHS) committee on scientific integrity voted to recommend that the PHS abandon its attempt to obtain an exemption to the law that allows individuals to see the contents of government files on them.

Under the proposed regulations, the National Institutes of Health and other research agencies in the health service would be granted an exemption to the Privacy Act to withhold the identity of whistleblowers. The act is intended to give individuals access to their own government records to ensure that they are accurate. In its request of 12 June for an exemption, the health service claims that such exemptions are routinely

granted to agencies that conduct criminal investigations.

But Estelle Fishbein, general counsel for Johns Hopkins University and a member of the misconduct advisory committee, believes that the proposed exemptions could violate a researcher's civil rights. If the exemption were to be granted, she points out, government health agencies would be allowed to withhold the identity of any informer. "An informer motivated by base motives [would] be accorded virtual immunity although his actions may set in motion government accusations, investigations, and hearings which will disrupt the personal and professional life of the scientist for years," she wrote in a comment to NSF. The Federation of American Societies for Experimental Biology and the Association of American Medical Colleges also oppose the proposed exemption.

Lyle Bivens, policy director for the PHS Office of Research Integrity, says that it is

unlikely that his office will change its mind. He points out that the Privacy Act exemption applies only to the initial, investigative, portion of the federal misconduct process. Once evidence of misconduct is found, the case is transferred to a court-like proceeding under an administrative law judge, who decides if sanctions are appropriate. In that proceeding, researchers have the right to face their accusers and to see the evidence against them.

Bivens says that the Privacy Act exemption would be used only when the identity of the accuser does not affect the case, as in a situation involving plagiarized material. But if a case rests on the testimony of a specific person, says Bivens, PHS officials would probably not withhold the person's name.

There is no guarantee that PHS officials would follow these internal policies, but Bivens argues that the risk of harm is slight given the different rules governing the administrative court proceeding. There is, however, one loophole: after an investigation finds evidence of misconduct and before the administrative court completes its proceedings, an accused scientist's name is put on a government list of scientists who are under investigation and should not be given grants. Although this is not considered a formal sanction, most scientists see it as a harsh penalty. If the health service is granted an exemption to the Privacy Act, researchers may soon find themselves on that list without knowing who put them there.

Christopher Anderson

Cold fusion produces heat but no papers

Tokyo. Advocates of cold fusion seem again to have forgotten the most elementary rule of scientific communication — publication. Last week, Japanese researchers at Nippon Telegraph and Telephone Corporation (NTT) generated tremendous heat among the country's media by announcing at a conference in Nagoya that they had found "undoubted direct evidence of cold fusion".

The statement is not new in the three-and-a-half-year history of the phenomenon. But, as has often been the case for supporters of a process claimed to have been discovered by chemists Stanley Pons and Martin Fleischmann, the NTT researchers have no paper, published or submitted, to back up their claim. The company's public relations department could not even provide an abstract for the announcement.

NTT scientists say that they have detected helium-4 from palladium electrodes soaked in deuterium gas and then heated in a vacuum. David Williams of University College, London, says that he is "underwhelmed" by their evidence and that similar findings have been traced to contamination from the atmosphere.

David Swinbanks

NSF, NIH differ on reporting conflicts

Washington. Controversy is a staple of US regulation of science, and conflict of interest is no exception. The Public Health Service (PHS), for example, was forced to withdraw proposed regulations published a few years ago and is now ruminating on a new set. The same angry reaction from the community has now befallen the National Science Foundation (NSF) after the publication of its own proposed conflict-of-interest regulations earlier this year (see *Nature* **358**, 700; 1992).

NSF received more than 70 negative responses to its request for comments; most focused on a proposed requirement that researchers disclose any potential financial conflicts to the agency when they apply for grants. In contrast to the NSF proposal, the current PHS draft regulations would require researchers to disclose their financial holdings only to their own institutions, which would then examine them for potential conflicts and certify to the federal agencies that none exists. Many of those writing to NSF were protesting against the fact that its proposed rules were different from the proposed PHS rules and would require additional paperwork.

Why not have the government speak with one voice? The answer is not reassuring. NSF did not follow the PHS rules because PHS would not share its draft of proposed regulations. PHS cited the need for confidentiality, but the argument would have carried more weight if the document was not widely available: half the universities in the country had already obtained a copy, and the University of Michigan even prepared a point-by-point comparison of the two proposals.

Meanwhile, Congress is threatening to muddy the waters. Last week the House of Representative's committee on government operations released another report in its continuing series on "Is Science for Sale?". Although this report focuses on conflicts with foreign corporations, it also recommends that PHS should embrace the NSF model and that researchers should be required to disclose conflicts when applying for federal funds.

Other legislators intend to take matters into their own hands. Several representatives, including John Dingell (Democrat, Michigan), the chairman of the House Energy and Commerce Committee, want researchers to disclose any relevant financial holdings in papers or talks based on federally funded research. The legislation, to be included in the same reauthorization bill for the National Institutes of Health that contains language lifting the current ban on fetal-tissue research (see page 761), is expected to be introduced in January and could be law by the spring.

Christopher Anderson

Japanese face stiff barriers in recycling of nuclear fuel

Tokyo. A proposal by Japan's leading nuclear power organization to recycle nuclear fuel to reduce the possibility of plutonium being diverted to nuclear weapons (see *Nature* 359, 663; 1992), is an old idea that faces formidable economic and technical barriers. But Japan's commitment to use plutonium regardless of cost (see story at right) may bring it within reach.

The idea is to incorporate into reprocessed plutonium fuel those radioactive transuranic elements, such as americium, neptunium and curium, normally discarded as high-level radioactive waste. The president of the Power Reactor and Nuclear Fuel Development Corporation (PNC), a semi-government organization affiliated with the Science and Technology Agency, hinted at the proposal at a symposium in Tokyo earlier this month, and his comments were fleshed out last week by PNC officials.

The approach would make it much more difficult to divert the plutonium to the manufacture of nuclear weapons. Weapons require fairly pure plutonium (ideally only the plutonium isotope 239), an alpha-particle emitter that can be handled in glove boxes and encased in a light shell. But americium 241, the dominant isotope of americium in high-level liquid waste, emits gamma as well as alpha radiation and can be handled only with manipulators in 'hot cells' that have concrete walls several metres thick. Thus, plutonium contaminated with americium is not suitable for making bombs.

John Nedderman, a British nuclear power consultant in Tokyo, says that the PNC proposal is a throwback to the idea of "dirty" reprocessing proposed by US President Jimmy Carter as a way of discouraging the highjacking of nuclear fuel. Although PNC officials agree, they say that the Carter plan was intended to block the start of a prototype fuel reprocessing plant in Tokai north of Tokyo and that it was never carried out.

Several countries have considered incorporating transuranic elements into fuel. France, for example, is looking into separating transuranic elements from high-level waste in its next-generation reprocessing technology. Similarly, Japan has poured billions of yen into its OMEGA project, one aim of which is to develop techniques for separating transuranic elements from high-level liquid waste. But PNC officials say the present proposal goes beyond OMEGA by incorporating the transuranics directly into the stream for reprocessed fuel.

Apart from the necessary chemical extraction technology, the PNC proposal requires sophisticated robot technology for processing the much more radioactive material into fuel. "This is why the idea may please politicians but it is not at all popular with fuel manufacturers or reactor operators" says Nedderman. But PNC is a semi-government organization generously funded by the Japanese government that can afford to look into what others see as an uncommercial process. **David Swinbanks**

Japan committed to plutonium at all costs

Tokyo. Japan will pursue the development of plutonium as an energy source, regardless of cost, because of a desire to end its dependence on outside sources of fuel. That much was made clear last week by an official of the Science and Technology Agency who released details of the annual white paper (policy document) on nuclear energy prepared by the Atomic Energy Commission. Its commitment comes despite the current unfavourable economics for plutonium and concern that it could be diverted into nuclear weapons or released accidentally into the environment.

The white paper takes a broad look at nuclear power, including the dismantling of nuclear weapons in the former Soviet Union, development of nuclear power stations in Japan and the rest of the world, nuclear fuel recycling and research on fusion. But with Japan preparing to receive more than a ton of weapons-grade plutonium from a nuclear fuel reprocessing plant in France (see *Nature* 359, 663; 1992), plutonium dominated questioning at the press conference on the annual paper.

Shigeru Maeda, deputy director of the agency's office of atomic energy policy research, said that developing domestic technology to extract plutonium from spent nuclear fuel would give Japan its own "mineral mines" and reduce its dependence on imported fuel. Asked if Japan would use plutonium even if uranium is cheaper, Maeda replied that "if we only base the argument on cost, there is a danger that it may lead to the wrong conclusion...there might be a suspension of the [imported] supply of energy to Japan".

Japan has wanted to achieve energy independence ever since the United States and Britain turned off the taps of imported oil in 1941. The oil crises of the 1970s and the recent Persian Gulf war have only strengthened the country's resolve. Plutonium, although derived from imported uranium fuel, is "quasi-domestic", according to government officials, because Japan can continue to produce it from spent nuclear fuel even if uranium imports are cut off. At the same time, plentiful worldwide supplies of cheap uranium have undermined attempts in the West to develop plutonium and the fast-breeder reactors that run on the fuel.

Even the Japanese nuclear power industry has begun to voice doubts about the economics of plutonium and fast-breeder reactors. But cheapness and ready availability of imported uranium barely enter into the equation in the minds of Japanese bureaucrats and politicians, who prize above all a reduced dependence on foreign sources of fuel. **David Swinbanks**

NEWS IN BRIEF

Washington. More than 350 physicists based in the United States have signed a petition to the Chinese Minister of Justice urging the release of Liu Gang, a 30-year-old graduate student in physics jailed for his pro-democracy activities. The petition, which was sent to Beijing last week, condemns China for "routine torture and humiliation" of inmates at Lingyuan prison, where Liu Gang is jailed, and seeks to free those confined for their democratic beliefs. The petition is sponsored by the American Physical Society and the Committee to End the Chinese Gulag. **T.W.**

Washington. The US National Aeronautics and Space Administration (NASA) is once again accepting applications from scientists with small experiments to be conducted on future flights of the space shuttle. The so-called Get Away Special programme has placed 87 payloads on 18 missions in the past ten years for research from universities, industry and domestic and foreign governments. Prices range up to \$27,000

for a five-cubic-foot, 200-pound payload, although educational institutions can qualify for a substantial discount. To make a reservation or to obtain additional information, contact Robert Tucker of NASA's Office of Space Flight at (202) 453-2347. **J.M.**

Washington. The White House Office of Science and Technology has created an Intergovernmental Council on Science, Engineering and Technology (InterSET) to improve communications on technical issues between the federal government and state and local authorities. The 10-member panel will be chaired by Allan Bromley, the president's science advisor, and will include present and former public officials with backgrounds in science and engineering. The council, which held its first meeting last week, is intended to make available to more public bodies solutions to a range of technical problems — from siting water reservoirs to identifying people who collect multiple government subsidies. **J.M.**

US and EC launch separate foundations to help Russians

Washington & London. The US Congress and the European Communities have independently created foundations to support joint research projects with scientists in the former Soviet republics. Combined, the two foundations may spend as much as \$20 million annually on research, scientific conferences and travel, a tremendous amount in a region where a scientist can be supported for less than \$2,000 a year.

Earlier this month, Congress passed legislation creating the AmeRus Foundation, a nongovernment foundation whose initial \$25 million endowment was also approved as part of the 1993 military budget. The foundation expects to begin operations more quickly than a comparable government initiative, perhaps accepting applications as soon as the beginning of next year for joint projects between US researchers and those in the former Soviet republics. The foundation is modelled on one between US and Israeli researchers that was formed in 1977 and, like the US-Israel foundation, it will use an international peer-review committee to review applications.

Staff members of the House science committee chaired by US Representative George Brown (Democrat, California), who sponsored the legislation, say they were surprised at the success of the bill. Both the National Science Foundation and the State Department saw the foundation as competition for the US-Russian science

institute that the administration has been trying to set up for almost a year (see *Nature* 355, 756; 1992). But it was helped by bipartisan support and the active involvement of Al Gore (Democrat, Tennessee and the Democratic candidate for vice-president).

Last week, the European Commission (EC) approved ECU4 million (US\$5.2 million) to start a similar foundation promoting science in the states of the former Soviet Union. Once operating, the EC foundation will fund joint projects with European researchers as well as travel, workshops and seminars. In January, EC ministers are expected to approve a budget for 1993; if it is deemed successful, the foundation will become part of the fourth Framework programme.

Although the current plan is for the EC foundation to be run by the government, EC lawyers are exploring ways to make the foundation a private corporation, incorporated in Belgium, which would bring it closer to its US counterpart and speed up a review process that could add months to the awarding of funds.

EC officials hope eventually to support around 5,000 researchers or group leaders, the majority in Russia, representing the top 5 per cent of the scientists in the former Soviet Union. The Russian government plans to supplement the grants awarded by both the EC and US foundations.

Christopher Anderson & Ian Mundell

Scope and speed of NSF commission is criticized

Washington. Add D. Allan Bromley and the members of an external scientific panel advising the president to the list of people unhappy with the National Science Foundation (NSF)'s Commission on the Future of NSF.

The NSF commission (see *Nature* 359, 261; 1992) has become a target for researchers concerned that its report, due on 20 November, will suggest that NSF should cater more to industry. Their fear stems from comments made by Walter Massey, director of NSF, in an August letter announcing the formation of the commission, but the commission's first two meetings suggests that it has no such plans and, indeed, that it is eager to affirm NSF's role as the primary source of federally funded basic research.

Bromley, the president's science adviser, has a different bone to pick with the commission. He thinks it is overstepping its boundaries by examining overall federal policies towards science instead of confining itself to NSF. The former, not coincidentally, is part of the responsibility of the White House Office of Science and Technology Policy (OSTP), which Bromley directs.

Although the 1950 law creating NSF gave its advisory body, the National Science Board, the authority to look at national issues, Bromley says that NSF's first director, Alan Waterman, "deliberately avoided" such a broad mandate, choosing instead to strengthen the foundation. "What you're seeing is an attempt by the science board to reclaim that turf, and I think it's inappropriate", says Bromley.

Last week, at the same meeting in which Bromley made his comments, members of the President's Council of Advisors on Science and Technology (PCAST) questioned whether the 15-member commission could accomplish its task in such a short time (its report is due nine weeks after its first meeting). "How on earth can they come up with anything useful so quickly?" asked Mary Good of Allied-Signal Corporation, a former chair of the science board.

Massey says that he expects the commission "to assess the total federal environment" in the course of plotting NSF's future but that it is free to set its own agenda. The commission has no plans to meet OSTP officials or those from other federal science agencies.

William Danforth, chancellor of Washington University in St Louis and co-chair of the commission, says that the report "will leave lots of avenues unexplored" but that it is up to the science board to decide if additional analysis is needed.

Jeffrey Mervis

Buyer shows a fine eye for science



This 1842 Powell and Lealand compound monocular microscope fetched £22,000 in a Sotheby's auction earlier this month, a record at auction for a nineteenth-century microscope and well above the estimated sale price of £6,000 to £9,000. Auctioneers say that the market for scientific instruments, although quite healthy, has yet to interest the sort of investment buyers who sent art prices through the roof in the 1980s. Collectors of scientific instruments tend to have an appreciation of the technical capabilities of what they buy, and particularly value items made or used by their inventors.

Ian Mundell

Sizing-up the brain

SIR — Four points stand out from the recent work of J. P. Rushton on racial differences in brain size (controlling for body weight) that you deemed too problematical for scientific publication¹.

(1) The brain size/IQ link exists but is slight. (Only one modern study reports a correlation as high as 0.35 (ref. 2), and this involved just one of several possible indices of brain size).

(2) The brain size/race link also exists, but is slighter. (Using thousands of subjects, the Black-White brain size difference approximates a mere one-sixth of a standard deviation in males and one-thirtieth s.d. in females.)

(3) The brain size/sex link is substantial. (Males and females differ by roughly a full s.d.)

(4) The IQ/sex link is non-existent — as Sir Cyril Burt had recognized by 1912. (The only important sex difference in psychometric intelligence is that the male s.d. is wider by 1 IQ point³ — thus the often-talked-of male superiority in 'spatial intelligence' is minute⁴.)

These four propositions are unlikely to be embraced in any single, simple theory — as Rushton and Ankney largely admit in their (now published) reports^{5,6}. Moreover, these four propositions cannot attest, help explain, or even be squared with the substantial (1 s.d.) Black-White difference in IQ tests.

Surprisingly, however, Schluter⁷ tries to cast doubt on the brain size/sex link. (Contrary to his apparent intentions, to dispense with this link would remove the biggest current obstacle to any claim that racial differences in intelligence result from differences in brain size; but Schluter is undeterred.) Schluter's argument is flawed. Its key proposition is: "If men truly have larger brains for their body size than women, men should be shorter than women of equal brain weight." Thus Schluter, finding brain-weight matched men still taller than control women, takes the link between brain size and sex to be false. However, men could, of course, on average, both be taller than women and have larger brains than would be expected from their size alone — as evidently happens. Schluter's prediction should have been that, with brain weight matched, the sex difference in height would be reduced, but not, of course, reversed.

Altogether, nothing coherent or of any theoretical significance has been learned so far from Rushton's latest sally into the minefield of psychological race differences. But *Nature* seems resolved to strive for political correctness and to expect Rushton and others in the field to meet higher academic standards than their critics. At the same time, in the

heat of controversy over racial differences, Rushton's 'scientific' critics will sometimes advance arguments that are self-defeating if accepted, but embarrassingly illogical in any case.

Scientific racists, if there are any, can be well pleased that bias and unreason will be deployed against them from high places. To provide such satisfaction is, perhaps, the inevitable cost of declining to examine Rushton's work open-mindedly on its scientific merits.

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SIR — Despite statistical problems in the literature, one of which Schluter⁷ notes, there seems to be adequate evidence that women have a mean brain mass about 100 g less than men of the same height. This statement is based on direct comparisons of mean brain sizes for each sex in the same height group (for example Table 1 of ref. 8).

Correction for body size, within or among mammalian species, as with Jerison's⁹ extra neurons above maintenance, is nevertheless unsure. This is because we don't know the extent (if any) to which larger body size decreases the evolutionary cost of a brain larger than needed for maintenance, as a result of the brain being proportionally smaller. To the extent that such a decrease in cost occurs, allometric correction will be over-correction because allometry itself involves more than the maintenance level. It is therefore conceivable that larger mammals, among and even within species, are on the average more intelligent than smaller ones with the same departure from the trend line.

However, Haug¹⁰ showed that neurons are more densely packed in the cerebral cortex of women than of men; the total average number is not detectably different. Neurons are also more densely packed in humans than in other mammals, but larger brains of each human sex have less densely packed neurons than do smaller brains, although still a greater total number¹⁰. Neuronal branching has not been studied.

Some years ago¹¹ I estimated the sex-specific correlation between human brain size and measured intelligence to be 0.3, from indirect evidence. This has now been corroborated² by direct evidence from a small sample.

Whether there are differences in general or special intelligence as a result of the various aspects of sexual dimorphism in human brains can't be investigated from standard tests, because those questions on which one sex does better

are eliminated when the tests are prepared. Biology really isn't destiny, and dimorphism doesn't imply any overall inequality, but (as Haldane¹² realized) understanding their effects is necessary in order to deal with these effects in a rational way.

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SIR — Some of your correspondents on the race and sex dependence of brain sizes illustrate Aldous Huxley's definition of philosophy: the finding of bad reasons for things you believe for worse reasons. The most compelling reason for believing that the races and sexes have different mental characteristics is the fury that this suggestion arouses in the politically correct.

Every orthodoxy in history, from the Inquisition to Marx to Lysenko, has shown inspired flair in embracing, and seeking to enforce belief in, nonsense. I don't know how they do it, but they are wonderfully reliable. Any claim asserted with menaces by an orthodoxy can safely be taken as false.

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Breaking the code

SIR — "Daedalus" (*Nature* **358**, 22; 1992) defines a "random sequence", claims that *Nature* is a nonrandom sequence, and suggests a way to encode an issue of *Nature* by a terse programme. But let that issue include an article which lists a sequence which is "random" by Daedalus's definition. (Suppose the article is about random sequences and includes examples.) That issue of *Nature* will then be a random sequence because part of it is. So that issue of *Nature* itself will be unencodable.

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Global ocean monitoring strategy

SIR — We applaud Duarte *et al.*¹ for emphasizing the need to maintain broad-area coastal and ocean monitoring studies. But they do not discuss the need for scientific and pragmatic incentives to encourage countries to monitor marine ecosystems.

A new paradigm in ocean use was initiated in 1982 when the United Nations Law of the Sea Convention established exclusive economic zones up to 200 nautical miles from the baselines of territorial seas, granting coastal states the sovereign rights to explore, manage, and conserve the natural resources from an ecosystem perspective². Within and extending seawards beyond the boundaries of the zones are large marine ecosystems being subjected to increased stress from growing exploitation of fish and other renewable resources, coastal-zone damage, river-basin runoff, the dumping of urban waste and the fallout from aerosol contaminants. Global climate change has become a factor in the sustainability of biomass production in large marine ecosystems³. The rather large-scale fluctuations in marine biomass yields of large marine ecosystems over the past several decades, when considered in the light of growing concern over coastal pollution and habitat loss, are serving to accelerate movement towards the development and implementation of a coastal global ocean observing system⁴ to provide biological, physical and chemical data for the development of indices to monitor changing states of large marine ecosystems⁵. The indices should improve communication between scientists and resource managers, should help to implement mitigation strategies where appropriate, and should reinforce the need for the long-term (multidecade) ecosystem-wide monitoring programmes that, according to Duarte *et al.*¹, are being terminated in Europe.

Monitoring and research are activities with different purposes and aims. Yet there is no clear-cut boundary between them, and monitoring the marine environment and living resources provides data that are used as an important component of research on perturbations and driving forces in large marine ecosystems. Insight into the mechanisms behind the large-scale fluctuations in biomass yields of these ecosystems is a prerequisite for an improved management strategy based on ecological knowledge and the principle of sustainable use⁶.

The 49 large marine ecosystems that have been identified are located around the margins of the ocean basins and extend over coastlines of several

countries⁷. They are in regions of the oceans most affected by overexploitation, pollution and habitat degradation, and collectively represent target areas for mitigation effort. The Global Environment Facility of the World Bank, in collaboration with National Oceanic and Atmospheric Administration, Intergovernmental Oceanographic Commission, United Nations Environment Programme, Food and Agriculture Organization of the United Nations, Natural Environment Research Council, the Sir Alister Hardy Foundation for Ocean Science and the national marine resource agencies of several countries (for example, Belgium, Cameroon, China, Denmark, Estonia, Germany, Ivory Coast, Japan, Kenya, Korea, The Netherlands, Nigeria, Norway, The Philippines, Poland and Thailand) are reviewing proposals to support assessment, mitigation and coastal monitoring activities of marine ecosystems as proposed by Duarte and his colleagues.

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Storing plutonium

SIR — Your leading article “Why not plutonium?” (*Nature* **358**, 356; 1992) contains two conflicting messages: that plutonium should be burnt in fast-breeder reactors and that plutonium should be stored in ‘secure repositories’.

A third option, plutonium disposal, should also have been mentioned. No commercial need for plutonium as a fuel for nuclear reactors exists anywhere in the world today, nor is it likely to for some decades. It is cheaper and safer to produce nuclear electricity by burning enriched uranium. As plutonium is not an ‘economic resource’ in any conventional sense, it should therefore be treated as radioactive waste.

The argument for storing separated plutonium (currently about 300 tonnes in weapons and civil stocks worldwide) against future use is also weak. Current world nuclear capacity (about 340 GWe) can be sustained with known economic uranium resources for about a century. When and if the world’s nuclear power capacity grows so large that fast reactors are required, the plutonium needed for reactor start-up could be derived from spent fuel from light-water reactors. For instance, if a tripling of nuclear capacity occurs to about 1,000 GWe, and there is then a wholesale shift to fast reactors over, say, a 40-year period, 150 tonnes of fissile plutonium would be required annually to fuel these reactors, or 6,000 tonnes over 40 years. A conventional ‘thermal’ nuclear programme of 1,000 GWe would discharge about 120 tonnes of plutonium annually. On these scales, the fate of 300 tonnes of plutonium seems relatively trivial.

Storing hundreds of tonnes of plutonium for several decades is not, however, a trivial problem. Plutonium is a bomb material and will always remain a target for proliferators and terrorist threats. Its use, handling and transport should therefore be restricted to the bare minimum. Treating plutonium surpluses as waste, immobilizing them in glass (or some other suitable material), with a view to eventual disposal seems to us a prudent approach to a global security and environmental problem.

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Motive to sin

SIR — E. T. Rakitzis (*Nature* **359**, 9; 1992) misrepresents my position (**358**, 10; 1992). Contrary to his assertion, I do not believe (and did not say) that people commit fraud because the stakes are high. I said they commit it because it is rewarded (the rewards and stakes are remarkably low by the standards of the business world). Such people are certainly unsuited for any position of responsibility, in science or elsewhere. But they exist, and always will, and science should be run so that they do not have the motive to sin. This is not a rationalization of fraud; it is the suggestion that it is better not to test everyone’s integrity too often. More important, the system that rewards fraud by the unscrupulous also rewards fraud by the honest.

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The 'far east' of biological ethics

Darryl Macer

The ethics of biological research, and its general impact, are hotly debated in the West. Japanese silence on the issues is counterproductive. Will the Human Genome Project provide the catalyst for change?

DURING the past 30 years, everyone has become familiar with ethical issues arising from technological change. Although technological development in Japan since the end of the Second World War has been famously rapid, social change has not mirrored this. When it comes to medicine, for example, fear and suspicion of this paternalistic profession means that 'informed consent' is a little-known concept. The advent of reproductive technologies such as *in vitro* fertilization failed to raise widespread public debate. There are no guidelines on somatic gene therapy, although many have called for them. Will the human genome sequencing project provide a catalyst for general discussion?

Japan contributes about an order of magnitude less than the United States to the Human Genome Project, but about two orders of magnitude less to ethical research on the subject. In the United States, 5.2% of the National Institutes of Health's budget for 1992 has been allocated to research on ethical, legal and social issues and on education. In Canada the figure is 7.5% and in Europe a similar proportion is being spent. But in Japan the figure is less than 1%. The contrast can be seen clearly by comparing funding in 1992: in Japan there are two projects of ¥5 million (\$80,000) whereas in the United States expenditure is \$7 million.

In Europe, scientific research for the Human Genome Project received financial support only after a system was established for funding of research on the ethical issues. In Japan, the only reason any ethical, legal or social research was funded at all was because one scientist decided to allocate 1% of the budget received from the Ministry of Education to set up a study group to organize a seminar on the subject. The 1992 budget was spent on the seminar and publication of the proceedings in English and Japanese (ref. 1). In 1992, a second project was set up by the Ministry of Health and Welfare². This tiny beginning cannot begin to address the ethical problem as a whole.

There have been international bioethics symposia in Japan since 1980 (ref. 3), but despite the participation of some biologists there is still no interdisciplinary dialogue. Scientists in Japan who do see the relevance of ethical studies do not think they should be the responsibility

of natural scientists, but of social scientists or lawyers. But even if social scientists start such research, they may still be unable and/or unwilling to challenge the views of biologists or policy-makers. It is more difficult for non-academics to discuss the views of scientists, even about public policy issues.

In the West, scientists in general have become convinced of the need to gather data and do research on ethical, legal and social issues. The situation in Japan is more related to the structure of its society than to any difference in individual people's attitudes. This can be shown from the results of opinion surveys, for example, my study⁴ in which the public, researchers and school-teachers were asked to give their reasoning for their opinions on bioethical issues such as genetic manipulation. Many people simultaneously perceive both benefits and risks; opinions are independent of education, age or profession. The overall statistical results of many of the questions, and the reasoning expressed, were similar to those from surveys in New Zealand, Europe and the United States.

In the same survey I also found that Japanese scientists show less concern about ethical issues associated with animal experimentation, consistent with the lack of guidelines for such experiments. One reason for there being no guidelines is fear of bureaucratic legislation. Japan is a legalistic society, in that behaviour is regulated by law rather than by ethics, and although some individuals recognize the importance of ethical issues, there is no general willingness to build on this concern via self-regulation, teaching courses and so on.

Perhaps the most well-known difference between Japan and elsewhere is the issue of brain death. It has been said that Japanese people would not accept organ transplantation from brain-dead donors. However, in a 1990 opinion poll of 3,000 adults, 51% of the respondents agreed with donation of their relatives' organs, 31% were unsure and only 16% disagreed. In a similar survey, in 1984, 20% said they would disagree, while 48% agreed to donation⁵. Despite this, only kidneys and corneas are donated from cadavers at present. The view that the Japanese have special cultural barriers to such donations has been dismissed by sociologists and religious groups in the

country. A more serious doubt in the minds of some Japanese people is whether they can trust doctors. Even a committee set up by the Prime Minister that reported earlier this year on the subject and which recognized this problem, was itself closed to the public.

It is therefore not surprising that genetic counselling is also being introduced very slowly. The 1948 Eugenic Protection Act does not officially allow abortions of fetuses because of genetic disease or chromosome abnormalities, yet abortion is easily obtained. Like the brain-death issue, people think that Japanese have different views from Westerners, but in fact they seem rather similar statistically. Japanese people strongly support the use of genetic screening and gene therapy⁴. Genetic screening services are not commonly available or funded by national health insurance; many major medical schools still do not have a genetics department. There is a pressing need for education, and research is required on how to introduce counselling and to reduce the stigma associated with genetic disease.

Ethics demands the recognition of the autonomy of all individuals to make free and informed decisions providing that they do not prevent others from making such decisions. However, the structured paternalism of Japanese society is built on the idea that only the views of so-called experts (*sensei*) should be heard. It also means that their views should not be questioned, in accordance with the traditional, paternalistic Confucian ethos. The main theme of Confucianist ethics was the maintenance of moral discipline for the nation, society and the home⁶; this of course was to the benefit of people in authority. It is not surprising that many of the authorities in Japanese society still wish to promulgate the idea that the Japanese are different.

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Surmounting fluctuating barriers

The idea that particles can surmount the walls of potential wells to escape from them is so familiar that the complexity of attempts to broaden the problem is a surprise.

EVEN those whose belief in quantum mechanics is of the formal kind, like that of those who give Einstein full marks for precocity, but who have never sensed that curvature can stand proxy for gravitational force, are often persuaded by one revelation of the elementary textbooks: particles trapped (in the classical sense) in a potential well can, in reality, leak out. The emission of α -particles from neutron-rich radioactive nuclei is a practical demonstration of the phenomenon; classically, centripetal nuclear forces would prevent the α -particles from escaping, but in quantum mechanics, they can tunnel through the potential energy barrier.

An even more compelling proof that quantum mechanics describes the real world (if only because the constituency of chemists is probably larger than any other) is the demonstration (rigorously by Kramers, only in 1940) that the rate at which particles escape a potential barrier is exponentially related to its height, say E , and the temperature at which the escaping particles are maintained, say T , by a factor in which the exponent is $-(E/kT)$, where k is Boltzmann's constant.

Chemists are encouraged because this is precisely the dependence (in matters such as reaction rates) discovered empirically by Arrhenius in the nineteenth century. One minor difficulty is that classical kinetic theory predicts the same kind of dependence; the essence of quantum tunnelling is that even particles with insufficient energy can tunnel through barriers that would classically constrain them.

Nevertheless the idea that reaction rates are regulated by the ease with which quantum systems can surmount potential barriers has become generally acceptable. The standard textbook picture is that of two regions of space separated by a fixed potential barrier. The problem is to tell the probability that particles on one side of the barrier, whatever their energy (which may vary), will escape to the other.

So far, so good. But what will happen if the potential barrier is not fixed for all time, but rather is itself variable? Only a little imagination is required to suggest a host of circumstances in which these circumstances might apply. The potential barrier to the disassociation of a molecule, for example, may depend on the geometrical positions of atoms not directly involved in what may be simply the stretching of a chemical bond to destruction. Or, as much

to the point, an insulating layer separating two semiconducting layers may be subject to a bias that affects the ease with which electrons can tunnel through it. The fluctuating barrier is a concept whose time appears to have come.

Charles R. Doering and Jonathan C. Gadoua from Clarkson University at Potsdam, New York, have arranged just that (*Phys. Rev. Lett.* **69**, 2,318; 19 October 1992). Their calculation is strictly classical, with no quantum tunnelling, and might apply to the particles of a perfect gas enclosed in a one-dimensional box that itself encloses the potential barrier. To make the problem tractable (and there is a long way to go), the authors consider the most stylized of all possible potential barriers — a simple symmetrical triangular profile that puts a maximum of the potential energy at the centre of the one-dimensional box.

The problem is really a problem in diffusion. If the box contains an ensemble of freely moving particles which is nevertheless sufficiently dense to ensure that the movement of each is impeded by its neighbours, its momentum will on the average be proportional to the slope of the potential barrier (the driving force), but will be augmented by the impulsive forces from collisions with other particles. Although these are assumed to be entirely random in magnitude and direction, subject to constituting white noise, they may well be sufficient to carry a particular particle over the peak of the triangular barrier.

That is the classical problem-setting for an ensemble of particles in a box with a fixed potential barrier. How to make the barrier fluctuate? Doering and Gadoua use the simplest possible recipe. They allow for two alternative triangular potential barriers in their one-dimensional box, but let its height switch from one value to another at times that are inherently unpredictable, but which are determined by a single time-constant which is numerically defined.

Where is this leading them (or us, for that matter)? To a counter-intuitive conclusion, that is where. What would one expect? Suppose the parameter that matters is the average time it takes a particle to cross the barrier (or to reach its highest point, which is the same thing).

If the fluctuations between one form of the barrier and the other are relatively slow (compared with the characteristic time-scale of the particles), the average crossing

time would be the mean of the average crossing times over the two barriers separately; on the average, each should be in place for the same length of time, while the edge effects in which the dynamics appropriate to one condition gave particles extra freedom in the other would shrink to zero. But if, conversely, the barrier fluctuations are relatively fast, the edge effects are everything and the particles behave as if they are exposed to an average of the two extreme potentials. Because the crossing time is related to an exponential function of the energy, the two averages are not the same at all.

In many ways, this is the simplest version of the fluctuating barrier problem there could be. (The notion that the intervals between switches from one barrier to the other should be random is not a complication, but the opposite, allowing the parametrization, as the saying goes, of the state of the system by the ratio of two time constants.) The authors have carried out Monte Carlo simulations of what happens and have reported analytical results where they are attainable (by the aid of a what is no doubt a formidable amount of unrecorded algebra).

The upshot is that naive expectations are confirmed when the switching time between one barrier and the other is either very long or very short. But, in between, particles almost always more easily surmount the fluctuating barrier than simple averaging would suggest. Moreover, there seems to be a resonance phenomenon. Given some match between the switching time and that taken by a particle to surmount the barrier, the ease of transition is even greater than expected. Anthropomorphically speaking, this may mean no more than that the particles that more easily get through are those that are clever enough to wait until the barrier is lowered.

The importance of these calculation probably lies in the analysis of the stability of truly complex systems, but those with other interests will remark on two features of this calculations. First, it is remarkable, in this day and age, how simple are the systems that form the substrate of people's elaborate calculations. That is one measure of how far there is to go to the treatment of the real world. And then there is the question of when it will be possible to treat real quantum systems. On that score too, there is a rough road ahead.

John Maddox

Ecology of globular clusters

Douglas C. Heggie

BY looking at the statistics of giant clusters of stars in our Galaxy, Hut and Djorgovski, on page 806 of this issue¹, neatly sidestep a perennial drawback in astronomy — that all we see is a snapshot of the Galaxy as it is now — and give us a glimpse of evolution in action. From observations, with the simplest of theory, they discover the rate at which these globular clusters evolve and die.

There are over 100 globular clusters in our Galaxy, each containing typically half a million stars. They evolve through two kinds of gravitational interaction: that between the stars within them and that with all the other stars in the Galaxy. It had long been thought that these processes are almost independent; that internal effects drive clusters through so-called core collapse, whereas external effects simply prevent clusters from getting too big. Then theoretical work² showed that external forces actually speed up core collapse, a prediction which was borne out about ten years later, when it was found³ that the majority of clusters with collapsed cores are near the centre of the Galaxy, where external forces are strongest.

What Hut and Djorgovski have now done is to use the statistics of structural parameters of the uncollapsed clusters to predict the rate at which clusters are now passing through core collapse. The result depends on the original distribution of these parameters, which can be estimated from the most unevolved clusters, and on the numbers of clusters that are observed at present to be hovering on the brink of collapse. It is very pleasing that the number of the collapsed clusters is perfectly consistent with the rate at which clusters are found to be collapsing, about two every billion years or so. In a similar way they have been able to estimate the rate at which clusters are being destroyed by galactic forces.

The long-term effect of these processes is that the clusters we see now are just the remnants of a considerably larger population of clusters that once existed in our Galaxy⁴, the remainder

having succumbed to the destructive effects of internal evolution and galactic forces. This popular theory was one of the many issues discussed at two recent workshops*. There are difficulties, however. As S. van den Bergh (Dominion Astrophysical Observatory, Canada) pointed out, the distribution of masses of the survivors should depend on the strength of the external effects, whereas observations show that the characteristic

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Now you see it . . . 47 Tucanae is one of the hundred or so surviving globular clusters in our Galaxy. Its core has not collapsed, but the stellar density there is very high, and the cluster is rich in millisecond pulsars.

size of clusters varies little from one environment to another.

What of the surviving clusters? External forces not only govern their size but have greatly altered the statistical properties of the stars that remain in them. The most massive surviving stars sink to the centre, leaving the stars of lower mass near the outside, where they are most prone to removal by galactic forces. Observations and theoretical modelling by G. Piotto (University of Padua) and colleagues show that the distribution of stellar masses in clusters is indeed well correlated with the strength of the external effects.

What of the surviving stars in the surviving clusters? It used to be thought that the stars evolve in ways quite independent of the destructive dynamical processes going on around them. Now, however, observers and theorists are

faced with a growing body of evidence implying that life is not that simple. For instance, the predominant colour of the stars in the centre of some clusters differs significantly from the predominant colour elsewhere, and this generally occurs only in the clusters with collapsed cores (Djorgovski). An obvious explanation is the segregation of stars of low and high mass, but it does not work: there are types of stars which are depleted in the centres, but which have rather similar masses to other stars that are overabundant there. Some unknown density-dependent process is altering the appearance of the stars, and the relative abundance of stars of different colours (R. Buonanno, Rome Observatory). Collisions between stars are one process whose effectiveness depends on the density of stars in space. Apparently it occurs too slowly to explain the colour gradients (E. S. Phinney, California Institute of Technology), but it is thought to be responsible for at least some 'blue stragglers', stars that seem too young to exist in these old stellar systems.

European Southern Observatory

Besides blue stragglers, there are other kinds of stars which seem remarkably overabundant in globular clusters, including low-mass X-ray binaries, consisting of a normal star which feeds a stream of gas to a neutron star about which it orbits. Relatively copious numbers of millisecond pulsars, which are rapidly rotating, highly magnetized neutron stars, are also found in globular clusters. Unfortunately, there are several factors which make it harder to account for these objects in

globular clusters than in the Galaxy as a whole. One is that most neutron stars are thought to be born with high speeds, far above the escape velocity from the potential well of even the most massive clusters. Another is that an abundance of neutron stars is thought to require large numbers of primordial massive stars within the cluster; however, in forming neutron stars at the end of their lives such stars lose so much mass that the cluster expands, and becomes more vulnerable to destructive galactic forces. These factors stretch almost to breaking point current theories for the origin of millisecond pulsars. At the workshop in

* *Dynamics of Globular Clusters*, Berkeley, 15–17 July, 1992. *The Globular Cluster–Galaxy Connection*, Santa Cruz, 19–29 July 1992. Proceedings of each to be published by the Astronomical Society of the Pacific.

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Berkeley the hottest debates were those between C. D. Bailyn (Yale University) and S. Kulkarni (California Institute of Technology) on this issue.

The main lesson to be learned from these developments is that the system of globular clusters is like a galactic ecosystem. None of the important issues can be considered in isolation, because they are so closely interlinked: the galactic gravitational field influences the dynamical evolution, the evolution influences the

stellar density, and this in turn affects the ways in which individual stars and binary stars can evolve. Several pieces of this web of interactions have become clearer in recent years, and one of the most exciting challenges in the coming decade will be the task of delineating the entire network. □

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CELL BIOLOGY

Progress by poisoning

Dan Cutler

EXOTIC poisons have often been instrumental in revealing cellular and molecular phenomena. A new and particularly striking example is to be found in the paper by Schiavo *et al.* on page 832 of this issue¹. The authors describe experiments which show that tetanus toxin (TeTx) and botulinum-B toxin (BoNT) exert their deadly effects through the cleavage of one particular synaptic vesicle membrane protein, synaptobrevin-2.

These neurotoxins are structurally similar and are each synthesized as a precursor with low toxicity, which is processed to the active form of two disulphide-linked subunits. One subunit (the heavy chain) mediates binding to the cell surface, conferring target specificity, and facilitates entry of the other subunit (the light chain) into the cytosol where it causes the toxic effect. The different clinical effects of the two toxins reflect their different target cells: TeTx causes spastic paralysis by blocking presynaptic neurotransmitter release in the central nervous system, whereas BoNT causes flaccid paralysis by blocking acetylcholine release at the neuromuscular junction.

Recently, Schiavo *et al.* and others looked into the possibility — initially suggested by protein sequence data — that both toxins are zinc-dependent endopeptidases. The conserved zinc-binding motif characteristic of this activity² seems to be functionally important; TeTx (refs 3, 4) and BoNT (ref. 5) light chains both contain zinc. Chelation of the ion both blocks the toxic effect of TeTx on *Aplysia* neurons injected with toxin, and inhibits the proteolytic activity of the light chain *in vitro*⁴.

What is the target of this proteolytic activity? Schiavo *et al.* have now explored the possibility that it lies in the synaptic vesicle membrane. They incubated highly purified synaptic vesicles with the toxins and show that both cleave a membrane protein with the

electrophoretic mobility of synaptobrevin-2 (ref. 6). The cleavage produces two fragments with a time course matching that of the inhibition of neurotransmitter release in *Aplysia* neurons injected with toxin. Protein sequencing confirms that the protein affected is synaptobrevin-2, and that both toxins cleave at the same single peptide bond. A peptide corresponding to the cleavage site can also inhibit toxin function both *in vivo* and *in vitro*. So it seems that TeTx and BoNT block transmitter release by cleavage of synaptobrevin-2, and that this is the only target for these toxins.

Apart from the satisfaction of identifying the molecular target of two neurotoxins whose effects have been known for such a long time (the symptoms of tetanus poisoning were described by Hippocrates), these are the first toxins to be shown to exert their actions through effects on a synaptic vesicle membrane protein. For those in the business this is an exciting development — work on this membrane is already intense, and further clues to the function of its proteins will add impetus to the field.

The identification and characterization of the protein components of the synaptic vesicle membrane is proceeding at such a pace that it may become the first organelle for which all the integral membrane protein components are described. They may be divided into two main categories⁷: components that carry out the uptake and storage of neurotransmitter; and components that include those responsible for the membrane traffic of synaptic vesicles, including targeting, docking and fusion at the presynaptic plasma membrane, as well as the subsequent retrieval and reconstitution of the vesicle. These events also occur during vesicular traffic between other membranes, but the proteins under consideration are confined to neurons and neuroendocrine cells. They must therefore carry out aspects of these tasks

particularly associated with neural and neuroendocrine secretion, including the regulation of secretion in response to external secretagogue action, and the tight docking of synaptic vesicles at the plasma membrane (thought to contribute to the extremely rapid exocytic response to stimulation).

It is proving difficult to assign specific functions to members of this group of proteins, although there has been some progress. Of the three main members, the synaptotagmins (p65) have been implicated in synaptic vesicle docking and fusion⁷ (although a recent paper describes a neuroendocrine cell line without p65 that still has a fully functional regulated secretory pathway⁸), and synaptophysin (p38) is a candidate for the exocytic pore complex⁷. But there have been few clues to the function of the synaptobrevins, and the unequivocal demonstration that the proteolytic cleavage of synaptobrevin-2 abolishes synaptic vesicle function is a major step forward.

The findings of Schiavo *et al.* raise many questions. The structure of synaptobrevin-2, with the bulk of the protein on the cytoplasmic side of the membrane, suggests that it interacts either with the plasma membrane or with some cytoplasmic molecule. But if synaptotagmin is involved in docking, what might synaptobrevin-2 be needed for? If it is involved in targeting, then where do the synapsins, which mediate interactions between the vesicle and the cytoskeleton⁹, fit in? Whether this apparent confusion of overlapping functions reflects the extensive (and possibly cooperative) interactions that occur¹⁰ between synaptic vesicle membrane proteins, or whether we do not yet appreciate the full range of tasks carried out by this membrane, is not clear. But one thing is certain: identification of a few more toxins whose target lies within the synaptic vesicle would be very useful. In the meantime, sales of tetanus toxin and botulinum-B toxin will surely rise. □

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On prisoners and cells

Karl Sigmund

IN 1902, the Russian anarchist Kropotkin wrote a book on mutual aid in biological communities, feeling that Darwin's disciples tended to overlook its prevalence. This is no longer the case: altruism in a competitive world of replicators is now a favourite issue among darwinists, and the 'Prisoner's Dilemma' has emerged as the standard metaphor to model the conflict between mutual support and selfish exploitation. This model gains new perspective in two papers by M. Nowak and R. M. May, one on page 826 of this issue¹ and one due to appear in the *International Journal of Bifurcation and Chaos*².

In the Prisoner's Dilemma game, two players are engaged in a joint venture. Both have the options to cooperate or to defect (not cooperate). If both cooperate, they obtain more than if they both defect. But a player defecting unilaterally gains still more, while the cheated partner receives even less. Defecting is therefore the better choice, no matter what the other player does. If the payoff is reproductive success and strategies are inherited, defectors are bound to take over. Where does this leave mutual aid?

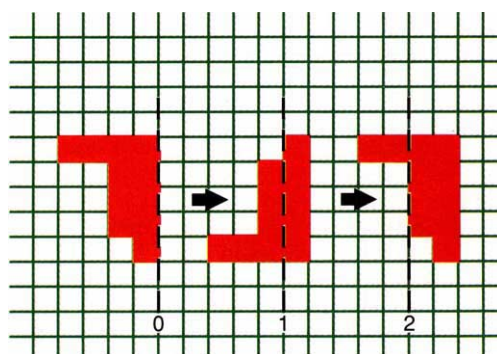
Repetition offers one way out of this impasse, as has been stressed by Axelrod and Hamilton^{3,4}. If retaliation is to be expected, it pays to refrain from defecting first because the bonus obtained by cheating in one round is offset by the risk of losing the partner's continued support. In order to retaliate in the iterated Prisoner's Dilemma, one has of course to recognize the other player. Defectors thrive in anonymous crowds, whereas mutual aid may be frequent between neighbours.

Roughly speaking, then, cooperating is good, but exploiting is better, as long as one can get away with it. Someone who is stuck in one place cannot get away with it, and therefore risks reprisals. But as Nowak and May point out, territoriality works even when no follow-up encounter is expected: it makes cooperation a viable option for the simple, single-shot Prisoner's Dilemma.

Indeed, imagine a population of players distributed on the squares of a chess board. Each player interacts only with the immediate neighbours. In the next generation, the square is inherited by whoever won most — neighbour or previous owner. Clearly, a lone cooperator will be exploited by the surrounding defectors. But four cooperators in a block can hold their own, because each interacts with more cooperators than a defector, as an 'outsider', can reach. If the bonus for cheating is not too large,

clusters of cooperators will grow. Of course, lone defectors will also do well: but, by prospering, they surround themselves with nasties and diminish their own returns.

Depending on the actual payoff values, the evolutionary dynamics can provide for various outcomes, but the usual result is regularly or irregularly fluctuating mosaics with both strategies holding on. In many cases, clusters of cooperators and clusters of defectors can both grow. If the initial conditions are random, computer simulations show an



Gliding on a wave of good will, a team of 11 cooperators moves through a world of defectors. Here, the pattern moves across a dotted line in three successive generations (time 0, 1 and 2).

endlessly milling spatio-temporal chaos, with metastatic tentacles flailing at each other. For symmetric initial conditions, a fantastic variety of patterns shows up, which could make a fortune for an enterprising tile manufacturer. That territoriality favours cooperation holds for many other neighbourhood geometries. It is likely to remain valid for random grids and stochastic transition rules, and for real-life communities; for example, the reciprocal altruism of aquatic cleaners and their obliging clients has been observed only among fish with fixed abode, not among those in the open sea.

Although Nowak and May are not the first to link loving one's neighbour with loving oneself (Matthew 22:29), they provide a new rationale for it, replacing the 'shadow of the future' (Axelrod's expression) by 'population viscosity' (a term due to Hamilton). Such a viscosity usually increases the probability that the coplayer is close kin, but Nowak and May make no assumptions about the genetic structure. The spread of successful strategies can be effected by cultural transmission just as well.

Spatial structures shoulder themselves into all kinds of population models nowadays, for instance, by stabilizing

host-parasite interactions or by protecting catalytic networks of replicating molecules; indeed, cooperation could be older than life itself. That apart, Nowak and May's combination of evolutionary game theory and cellular automata may well become a new fashion in computational mathematics. John von Neumann, who stood at the origin of both game and automata theory (and of the computer), would have enjoyed this union of his brainchildren.

Cellular automata⁵ provide a natural framework for evolutionary models with spatial structure. The best-known example is J. H. Conway's computer 'Game of Life'⁶. As discrete-time, discrete-space analogues of partial differential equations⁷, cellular automata are ideally suited for studying pattern formation and offer a vast field for mathematical exploration. The Nowak-May approach yields large classes of cellular automata, not just for the Prisoner's Dilemma but for other games such as 'Hawk-Dove'⁸ or 'Stone-Scissors-Paper'. Whereas Conway imposed arbitrary rules (after a lot of experimentation), the transition rules in the new automata emerge from evolutionarily relevant games in a natural way. Like the 'Game of Life', they exhibit glider structures (see figure) and a rich collection of other astonishing objects.

Few general principles are known for cellular automata, aside from a rough classification of their massively parallel dynamics of information transfer⁹, but it seems likely that some of Nowak and May's cellular automata are as complex as a universal computer. This would mean that, like 'Life', they contain patterns whose behaviour is on principle unpredictable. The possibility that the simplest conceivable two-player games can lead to fundamentally undecidable problems adds an intriguing dimension to Nowak and May's pulsating patterns of 'kaleidoscooperation'. □

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Antarctic ice sheets at risk?

David Sugden

GLACIAL deposits in Antarctica from 3 million years ago warn us of possible consequences of global warming, say Barrett *et al.* on page 816 of this issue¹. At that time, during the Pliocene, the global climate was a few degrees warmer than it is now, and the deposits indicate that the East Antarctic ice sheet was dramatically smaller, perhaps indicating an unstable response to climate warming.

For some years there has been a debate as to whether the East Antarctic ice sheet responds to warming in a measured, stable way or by unstable collapse. The problem is important because it concerns nothing less than the evolution of polar ice sheets and the global climate system. There are also serious implications for a world experiencing greenhouse warming. The ice sheet is larger than the continental United States and forms a convex dome rising to altitudes in excess of 4 km; it locks up a mass of water which, if melted, would raise world sea level by around 60 m.

The case for the instability of the East Antarctic ice sheet depends on the interpretation of a glacial deposit, the Sirius Group, which occurs throughout the Transantarctic Mountains. The deposit contains pieces of *Nothofagus* (southern beech), leaves and pollen². The nature of the vegetation and its incorporation in the deposit suggests that it has been overrun by glaciers advancing into a cool temperate environment, such as that now enjoyed by southern Patagonia. Crucially, the deposit also contains marine diatoms, certain species of which are thought to have existed only during the Pliocene 2–3 million years ago³. The implication is that the diatoms were growing in marine basins, and that sub-

sequently an ice sheet covered the basins and transported the marine deposits into the Transantarctic Mountains. The only realistic location for the marine basins is in the interior of Antarctica — hence the argument that the ice sheet must have been sufficiently small about 3 million years ago to expose much of East Antarctica to the sea (see map).

The significance of the new observations described by Barrett *et al.*¹ is that independent dating of volcanic ash confirms the dating that previously relied on biostratigraphic correlation. In a bore-

hollow has occurred since the Pliocene, and that the cooling associated with uplift could have triggered the growth of the East Antarctic ice sheet⁵. Also, it has been suggested that if, as forecast, global temperatures rise a few degrees to Pliocene levels, then the ice sheet can be expected to decay⁶.

The case for ice-sheet instability is, then, a strong one. But there is contradictory evidence, as aired at two recent conferences*. First, there is geomorphological evidence of prolonged polar desert conditions which is incompatible with the view that temperate forests thrived in the area during the Pliocene. G. H. Denton (University of Maine) and D. R. Marchant (University of Edinburgh) presented the results of laser

D. R. Marchant

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The Olympus Range and Shapeless Mountain, in the Transantarctic Mountains.

hole in shallow water just off the terminus of Ferrar Glacier in the Dry Valleys is a sequence of glacial and marine deposits containing a zone of diatoms similar to those in the Sirius deposits, including *Thalassiosira vulnifica* which is biostratigraphically dated at 2.2–3.1 million years old. A 30-cm layer of volcanic ash in this zone gave an age of 3 million years, confirming the biostratigraphic dating.

The possible collapse of the ice sheet in the Pliocene has been taken up by other branches of Earth science. High Pliocene sea levels in the eastern United States have been related to high global sea levels caused by the melting of the East Antarctic ice sheet⁴. The presence of temperate vegetation in the cold, high-altitude climate of the Transantarctic Mountains has been used to argue that significant tectonic uplift of the moun-

tain ranges has occurred since the Pliocene, and that the cooling associated with uplift could have triggered the growth of the East Antarctic ice sheet⁵. Also, it has been suggested that if, as forecast, global temperatures rise a few degrees to Pliocene levels, then the ice sheet can be expected to decay⁶. The case for ice-sheet instability is, then, a strong one. But there is contradictory evidence, as aired at two recent conferences*. First, there is geomorphological evidence of prolonged polar desert conditions which is incompatible with the view that temperate forests thrived in the area during the Pliocene. G. H. Denton (University of Maine) and D. R. Marchant (University of Edinburgh) presented the results of laser

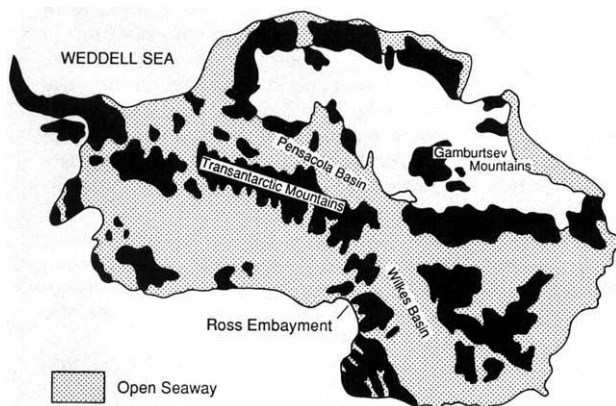


Diagram of the hypothesized deglaciation of Antarctica during the Pliocene, showing possible open seaways and marine basins. Barrett *et al.*¹ contend that marine diatoms from these basins were subsequently deposited throughout the Transantarctic Mountains by an expanding ice sheet.

* *Cenozoic Glaciations and Deglaciations*, London, 18 September 1992. *Cenozoic Climate Change and Tectonics*, Haarlem, 28 September–1 October 1992.

tion if it had occurred under cool temperate Pliocene conditions.

A second argument for stability comes from sea cores. J. P. Kennett (University of California, Santa Barbara) claimed that there is no sign in them of the change that would have accompanied the collapse of the East Antarctic ice sheet. Oxygen isotope ratios, strontium isotope ratios, the pattern of biogenic sediment and ice-rafted debris distribution all show little change through the Pliocene and point to no more than limited fluctuations of the East Antarctic ice sheet.

A third argument for stability is glaciological. Ice sheets in dry, cold, polar environments are restricted by a shortage of snow rather than by warm temperatures. In such environments, an increase in temperature of a few degrees is accompanied by more snowfall and an expansion rather than a decrease in the size of the ice sheet. P. Huybrechts (Free University of Brussels) used glaciological modelling to show that an increase of around 20–25 °C would be necessary to remove the ice sheet from the interior of East Antarctica. Furthermore, warming by up to 5 °C would lead to an increase in ice volume as a result of the increased snowfall that would accompany warming in a polar desert climate. The implication is that the ice sheet could not have disappeared during the modest warming of the Pliocene; rather, it is likely to have been bigger than it is today.

At present, it is difficult to reconcile the contrasting views about the stability or instability of the East Antarctic ice sheet. The impasse will be solved by careful scrutiny of the assumptions underlying the alternative hypotheses. Barrett *et al.*¹ remove one important uncertainty, that of the age of the diatoms. But there remains the problem of whether the diatoms are the same age as the terrestrial vegetation and glacial deposit in which they lie, and whether they were emplaced by ice. If not, then the Sirius Group deposits could be very old indeed and could reflect the onset of Antarctic glaciation in the early Cenozoic, about 40 million years ago. □

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Flowering inferno

TALK of pollution, and the instant thought is of smog over Los Angeles, or of floods of industrial effluent. But it is becoming increasingly apparent that ordinary agricultural practices are also villains in the piece. A third of anthropogenic carbon dioxide and carbon monoxide comes from biomass burning, participants concluded at a conference held two years ago. Although that leaves two-thirds coming from burning of fossil fuel, it was clear that there is a big gap in our picture of the factors that

Fire—Atmosphere Research Initiative) burnings, two destroying a little more than 3,000 square kilometres each and four smaller ones, were timed to start as the US National Oceanographic and Atmospheric Administration's NOAA9 satellite was flying overhead. At the same time, a pair of helicopters took coordinated measurements of the fire temperature and the chemistry of the air immediately above the blaze; a Cessna sampled the air tens of kilometres downwind and a DC3 took



Savanna ablaze in Kruger National Park, South Africa, during the SAFARI experiment.

could cause global climate change, and the decision was to rectify matters.

On page 812 of this issue, D. R. Cahoon and colleagues use military satellite images to find the patterns of grassland burning in the African savanna belts: grassland burning consumes three times as much biomass as does the much better known rainforest destruction, and two-thirds of all savanna is in Africa (10 million square kilometres). The grassland is burnt back regularly to make way for new shoots, as otherwise the land would quickly become unusable for grazing.

The main determining factor in the extent of burning is season — fires are most prevalent in dry conditions. At this time of year, large tracts of coastal southern Africa, from Namibia round to Tanzania, are aflame, but by January the destruction will have moved north and then inland to the sub-Saharan savannas of North Africa.

As a second prong of the new work, over 180 scientists from 13 nations converged on South Africa in September to study at first hand the atmospheric and chemical effects of fires started deliberately in Kruger National Park. The SAFARI (Southern African

further samples hundreds of kilometres downwind (as far as the Atlantic coast). But most workers were involved in ground-level observations. Not only was the amount of visible biomass before and after the fires measured, but even the effect of microbial biomass in the soil, a significant source of nitrogen oxides, was determined.

Of particular concern — greenhouse gases notwithstanding — is the role of the products of biomass burning in producing ozone in the troposphere: although ozone is beneficial in the stratosphere, shielding us from solar ultraviolet radiation, down here in the troposphere it is both a poison and another greenhouse gas. The conference two years ago determined that 38 per cent of tropospheric ozone is generated through biomass burning, and satellite mapping has revealed large plumes of ozone extending over the South Atlantic.

The masses of data collected will take months to digest — the participants in SAFARI will gather at the end of next May to pull all the threads together. But the hope is that a giant piece in the global environmental debate will then be put into place. R. P.

Joel S. Levine, NASA Langley Research Center

Variation is now the theme

P. N. Goodfellow

ON page 794 of this issue¹, Weissenbach and colleagues unveil "A second-generation linkage map of the human genome". Their paper appears less than a month after the publication of another comprehensive genetic linkage map, in *Science*², and together the two show that the Human Genome Project is building up an unstoppable momentum.

In genetics you study phenotypes, and in human genetics the phenotypes are often diseases. Genetic maps are used to associate genes with phenotypes, so that human maps are of great medical importance. The first attempts to create maps were made by a few dedicated scientists working with an incomplete set of tools. Most regarded those geneticists with the view my mother had of people who wore beards and ate yoghurt: they were eccentrics. Things have changed, and fast. Map construction is now big science, run by scientist-administrators and factory managers, and the two new reports attest to the success of that approach.

Why are these maps cause for celebration? Because it has not previously been possible to produce complete maps of the human genome, as distinct from those of model organisms. The techniques of molecular biology have been needed to extend the repertoire of known natural variability in the human genome beyond that provided by known genetic diseases and other recognizable phenotypic characteristics, such as blood groups and HLA types.

Making a map of the genome entails estimating the distance between pairs of genes or other genomic landmarks, which (classically) must be done by inference from comparison of the occurrence of recognizable phenotypes in successive generations. When the genes are on different chromosomes, they segregate into germ cells independently. But genes on the same chromosome do not. If they are close together, they behave as if they are linked; further apart, and the linkage may be disrupted by the process of recombination at meiosis. So the distance between genes is a function of the probability that linked genes (or other genomic landmarks) will be separated by recombination. The unit of measurement is the centimorgan (cM), corresponding roughly to a 1% frequency of recombination (and in humans, on average, to 10⁶ base pairs of DNA).

These principles have been known for over 50 years, but until now it has not been possible to use them to produce detailed maps of the human genome. A genetic marker must exist in two or more

polymorphic forms reflecting different alleles of the underlying gene. Classically, the markers used in map construction have been either proteins or indirect products of genes, but protein polymorphism is usually low. In experimental animals this problem can be surmounted by enforcing specific matings on captive populations; obviously the same could not be done in human genetics. The solution was found by exploiting DNA sequence variation.

The simplest form of sequence variation occurs every few hundred base pairs

can have higher values. Heterozygosities in excess of 70% are common for tandem repeats. Minisatellites are made of arrays of a core sequence of 10 base pairs or more; unfortunately their distribution is biased towards chromosome ends⁹. Microsatellites, composed of mono-, di-, tri- and tetranucleotide repeats, are more randomly distributed, very frequent and can be scored by PCR (polymerase chain reaction). The high levels of heterozygosity and the ease of automating PCR makes them ideal markers for constructing genetic maps.

Armed with RFLPs, several groups started constructing maps of the genome, but (in the words of E. Robson) "private maps like private armies induce fights". In a contribution to world

peace, Jean Dausset created CEPH (Centre d'Etude du Polymorphisme Humain) using money from his Nobel prize and a large legacy. Workers at this centre collected a reference panel of human families and made DNA from them available to mapping community¹⁰. This made all contributions to the mapping effort additive, and the end result of this global collaboration is reported in *Science*². The National Institutes of

Human genome count-down

1993. A complete contig of the human genome in yeast artificial chromosomes. A 1-cM genetic map. Integration of the two maps and correlation with the cytogenetic map.

1994. 1 megabase of contiguous human DNA sequenced from the Huntington region of chromosome 4 and from Xq28. A cosmid contig of the genome constructed using the yeast-artificial-chromosome contig as a template.

1996. 10 megabases of sequence from Xq28 and 21q.

1997. Chromosome 21 sequenced. Estimates of the number of genes in the genome increased.

1999. Complete sequence of the genome.

in the genome³ and can be detected by Southern blotting. Immobilized genomic DNA is hybridized to a cloned probe after cleavage with a restriction endonuclease and size separation by electrophoresis. The size of the fragments detected depends on the spacing of the sites of cleavage; deletions, insertions and mutation, causing loss or gain of a cleavage site, results in a size shift. These molecular phenotypes were given the acronym RFLP (restriction fragment length polymorphism). Despite the importance of RFLPs as markers^{4,5}, they suffer from several drawbacks. The level of variation associated with most RFLPs is low and is usually limited by the number of alleles. In addition, Southern blotting is time consuming and not well suited to automation.

The problem of low levels of polymorphism can be solved by scoring variation in the number of repeats found in tandem arrays in microsatellites⁶ and minisatellites⁷ (minisatellites are also called VNTRs or variable number of tandem repeats⁸): the repeat number is often highly variable and is associated with several alleles. One measure of variation is the chance of an individual being heterozygous (that is, having two different alleles of a gene): the maximum heterozygosity for a gene with two alleles is 50%; genes with several alleles

Health and CEPH collaborative group have constructed a map consisting of 1,416 loci spread over 90% of the genome. This remarkable achievement involving hundreds of scientists is not, however, the last word in genome mapping. Most of the markers used are RFLPs and only 205 of the markers used are microsatellites with heterozygosities of more than 70%.

Supported by public subscription, via the medium of television, the French have erected Genethon, a factory for human genetics. In this issue, Weissenbach, Lathrop and colleagues from Genethon describe a map based on 792 (C-A) repeat microsatellite markers of which almost 600 are highly informative¹. The markers were isolated and the map was constructed by a group of about 20 people using the organizational methods pioneered by Henry Ford. Microsatellite markers, for the map, were identified by screening a clone library of small fragments of human DNA. Clones positive for (C-A)_n repeats were sequenced and pairs of PCR primers chosen which flank the tandem repeats. The markers were assigned to individual chromosomes using somatic cell hybrids and then tested for linkage analysis on a panel of eight CEPH families. The final map is estimated to span more than 90% of the human genome

with an average distance between adjacent markers of about 5 cM. Both the NIH/CEPH consortium and the Genethon group have used CEPH families, which will make combining the two maps straightforward.

The creation of a high-density meiotic map of the human genome is good news for the Human Genome Project, as is the recent generation of a microsatellite-based map in the mouse¹¹ and the construction of large yeast-artificial-chromosome contigs of human chromosomes (described in New and Views¹²). At a practical level these advances will help the mapping of genes causing less common genetic diseases, allowing for prenatal diagnosis and eventual gene cloning. A high-resolution map is also required for identifying genetic components of complex common diseases, such as cancer, behavioural disorders, heart disease and diabetes. Solving these problems will benefit everyone.

This is not the first time that maps of the human genome have been heralded in the popular and scientific literature; the cynic may ask, "Is this the last?" The answer is an emphatic "No". The factories are running and need to generate new products. In the next year, the landmark publication will describe integration of the genetic map with cytogenetic and physical maps of the genome. By the end of 1993, I predict that Weissenbach and colleagues will produce a map with an average marker spacing of less than 1 cM; this is well in advance of expectations. A contig of the genome in yeast artificial chromosomes and an integrated genetic map will provide a sound platform for sequencing the genome: this will be finished by the end of the decade (see box).

The biological sciences are learning that goal-oriented science based on existing technology is best done in factories and can deliver excellent value for money. This should not, however, be allowed to overshadow the less quantifiable contributions made by curiosity-driven science. Both have a place and I, for one, still have a beard and eat yoghurt (sorry mother). □

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A new hydrocarbon cage

Leonard F. Lindoy

In a striking parallel to the development of the fullerenes, F. Vögtle, J. Gross, C. Seel and M. Nierger of the University of Bonn report an elegant synthesis for an almost spherical cage of composition $C_{36}H_{36}$ (*Angew. Chem. Int. Ed. Engl.* **31**, 1069–1070; 1992). They name the new species spheriphane.

The synthesis of spheriphane involves a kind of molecular fold-and-paste exercise, not unlike making paper sculptures. The immediate, extended precursor (**1** in Fig. 1) to $C_{36}H_{36}$, which is made in six synthetic steps, can be wrapped up by either of two routes (Fig. 1) of which the second, involving intermolecular three-fold coupling, is the more efficient. The authors find that the 1H and ^{13}C NMR spectra of $C_{36}H_{36}$ are both simple, confirming the symmetrical nature of the product. The 1H spectrum shows only two signals, corresponding to the aromatic and benzylic protons, respectively, while the ^{13}C spectrum consists of three peaks — arising from the three carbon environments present in the molecule.

The molecular skeleton is flexible enough to allow rapid twisting of the ethane linkages connecting the aromatic rings; this results in the respective methylene hydrogens appearing equivalent in the 1H NMR spectrum, even at temperatures below $-90^\circ C$. Such flexibility is a potentially important characteristic of cage molecules if they are to be used as hosts for encapsulating suitable guests; spheriphane is large enough to admit small metal ions. Some flexibility will normally aid the passage of a guest into and out of the central cavity.

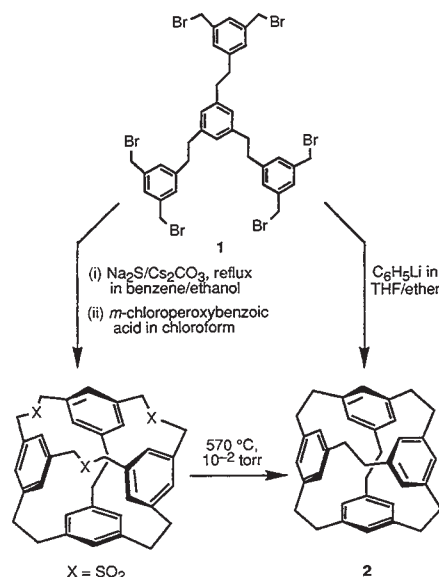


FIG. 1 The final steps in the synthesis of $C_{36}H_{36}$ (**2**).

Spheriphane can be crystallized from solution as its chloroform adduct. An X-ray structural analysis of this again confirms the almost spherical form of the cage and shows that the centroids of the four benzene rings form a slightly compressed tetrahedron (Fig. 2). The radius

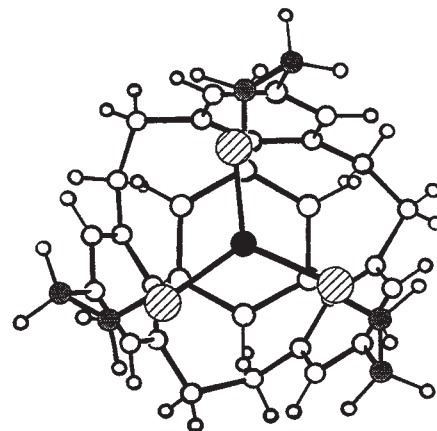


FIG. 2 The X-ray structure of the chloroform adduct of $C_{36}H_{36}$ viewed along the symmetry axis. The relative orientations of the hatched and shaded carbon atoms illustrate the helical arrangement present in the framework of the cage.

of the central cavity of this cage, at 284 picometres, is significantly less than in C_{60} (351 picometres) (J. M. Hawkins, A. Meyer, T. A. Lewis, S. Loren & F. J. Hollander *Science* **252**, 312–313; 1991), but is larger than in the previously reported cage $C_{20}H_{20}$, dodecahedrane (216 picometres) (J. C. Gallucci, C. W. Doecke & L. A. Paquette *J. Am. chem. Soc.* **108**, 1343–1344; 1986). Interestingly, the chloroform molecule in the crystallized form is outside the cavity but lies on the adduct's symmetry axis — it is held in place by an attractive interaction between the hydrogen and the π system of one of the aromatic rings.

A novel feature of the cage structure is the helical arrangement formed as part of the spherical framework (Fig. 2). Such a feature will give rise to a chiral environment within the central cavity; unfortunately, the radius of the spheriphane cage appears to be too small for it to act as a receptor for the chiral discrimination of small molecules.

Reaction of $C_{36}H_{36}$ with silver trifluoromethanesulphonate in tetrahydrofuran readily leads to a solid complex having a 1:1 (metal:cage) stoichiometry. Because of low solubility, this species has not been fully characterized. However, the incorporation of the silver ion in the cavity is certainly a possibility; alternatively, the ion may be externally coordinated to the surface of the cage.

RÉSUMÉ

The authors attempted to remove hydrogen from $C_{36}H_{36}$, using palladium-charcoal heated to 500 °C, but they were unsuccessful. Full dehydrogenation to give an all-carbon cage related to the fullerenes is, in any case, not expected as the bonding pattern in such a product seems almost certain to be unfavourable. The reverse process of reducing $C_{36}H_{36}$ in the presence of a platinum catalyst led to the aromatic rings being successively hydrogenated up to and including the formation of $C_{36}H_{60}$.

This synthesis is an important milestone in the continuing development of supramolecules incorporating three-dimensional cavities. The aim is to add

to the range of synthetic strategies and precursor fragments, so opening the way to new families of cages enclosing cavities of various sizes and electronic nature. Molecules of this type — with tunable, hydrophobic interiors — widen the options for producing new classes of receptors. For example, they appear to offer very considerable potential for the selective capture of individual ions and molecules in a range of applications which includes pollution control. □

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CLIMATE CYCLES

Upset for Milankovitch theory

Wallace S. Broecker

ONE of the fundamental tenets of palaeoclimate modelling, the Milankovitch theory, is called into doubt by isotope analyses of a calcite vein, just reported in *Science* by Winograd and colleagues^{1,2}. The theory, which is backed up by a compelling bank of evidence, suggests that the ice ages are driven by periodic variations in the Earth's orbit. But the timing of the ice ages determined, with unprecedented accuracy, in the new record cannot be reconciled with the planetary cyclicity.

The idea behind Milankovitch theory is that changes in the tilt of the Earth's rotation axis and in the point in the annual cycle of the Earth's closest approach to the Sun alter the way solar radiation forces the climate. An exceptionally good match exists between the peaks and troughs in the radiometrically dated ice-volume record and those expected from variations of summer insolation in the Northern Hemisphere mid- to high latitudes. Specifically, the oxygen isotope composition of shells of marine foraminifera has varied with the precession of the Earth's spin axis. Indeed, the magnitude of the climate changes so revealed were in direct proportion to the eccentricity of the Earth's orbit³.

Winograd and colleagues' evidence also turns on oxygen isotope data, this time from vein calcite coating the hanging wall of an extensional fault at Devils Hole, an aquifer in southern Nevada. In 1988, the authors⁴ published a date, 145,000 years, based on ^{234}U - ^{230}Th dating for the end of the penultimate ice age (Termination II), marked by an increase in the ^{18}O to ^{16}O ratio, a change taken to mirror an increase in local precipitation. Although the date was only 17,000 years earlier than the previously accepted date of 128,000 years, if

correct, this change is enough to bring the Milankovitch mechanism into serious doubt.

Not surprisingly, palaeoclimatologists refused to take this lying down. Because of the long traverse time between the source of the Devils Hole signal (the aquifer recharge zone) and the site of the recorder (at the aquifer exit) the validity of the match between the ^{18}O record for the Devils Hole calcite vein and that for marine foraminifera was doubted. A finite age was obtained for the outer surface of the Devils Hole calcite vein, bringing the ^{230}Th - ^{234}U chronology into question. The accepted marine chronology was defended on the grounds of a 124,000-year ^{230}U - ^{234}U age for coral reefs formed during the sea-level maximum of the last interglacial, providing a firm anchor point for the timescale. Nevertheless, no satisfactory alternative explanation for the Devils Hole record could be given.

Because of this, the publication of further results was eagerly awaited. The two new papers^{1,2} provide additional evidence which undermines much of the earlier criticism. The ^{18}O record in Devils Hole has been extended back two more full climatic cycles to about 550,000 years. The match with the marine ^{18}O record remains strong¹, demonstrating that the oxygen isotope composition of Devils Hole calcite has changed in harmony with that in deep-sea foraminifera. Uranium series measurements made by mass spectrometry² verify and make more accurate the earlier chronology. Further, ^{234}U - ^{238}U measurements on water currently upwelling in Devils Hole confirm the validity of the 60,000 year age for the outermost surface of the vein calcite. These measurements yield a completely

Dead good

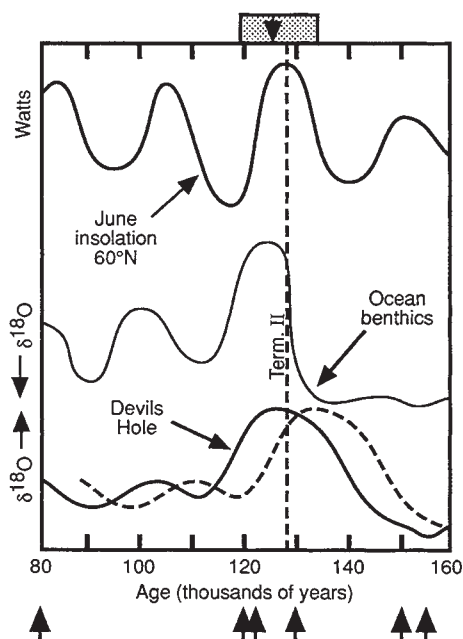
LUBBER grasshoppers are not palatable — in laboratory experiments, 21 species of bird and lizard refused to eat them, vomited if they did or, in the case of one unfortunate meadowlark, died. But loggerhead shrikes in Florida can get round these formidable chemical defences (R. Yosef & D. W. Whitman *Evolut. Ecol.* **6**, 527–536; 1992). The birds often impale their prey on thorns or barbed wire, either as a food cache or in courtship. Now it seems that this behaviour has another function. Although shrikes cannot eat fresh lubbers, they will consume them after corpses have aged for a day or two (by which time, presumably, the toxins have lost their potency). In nature, as in human affairs, no defence is perfect.

Growing stronger

IN the quest for ever stronger materials, F. T. Wallenburger and P. C. Nordine have developed a way of growing boron fibres that rival commercial carbon and silicon fibres in strength (*Mater. Lett.* **14**, 198–202; 1992). Until now, no reliable method had been found to do this (except for growing the boron around a filamentary tungsten substrate, which left the product too thick for its optimum material properties). But Wallenburger and Nordine have succeeded in growing fibres just 6 μm across and up to 2 m long by irradiating and dissociating high-pressure diborane (B_2H_6) gas, in hydrogen, with tightly focused laser light. At first the laser spot is kept aimed at a simple substrate surface; but once the boron growth has been nucleated, the substrate is drawn away as fast as the filament grows, at around 0.5 mm a second. That may seem slow, but only single-crystal silicon carbide whiskers have a higher tensile strength and they can be grown to only a few millimetres long.

Skin deep

IN a dazzling paper published in 1902 Rudyard Kipling sketched out his *Just So* hypothesis of how the rhinoceros got his skin. The aims of R. E. Shadwick and colleagues (*Proc. R. Soc.* **B337**, 419–428; 1992) are more modest. On the death of Duncan, a white rhino at Calgary zoo, they took skin samples and subjected them to structural and mechanical tests. It turns out that rhino skin, especially on the back and flanks (the areas most exposed during intraspecific fights), is not just thicker than that of other animals. Rather, it has properties intermediate between skin and tendon, making its strength, stiffness and work of fracture extremely high. The skin is comparatively easily torn which, say the authors, may provide a defensive advantage, in that in combat the result is more likely to be gashes than lethal punctures.



Comparison of the histories of June insolation at 60°N and the oxygen isotope records ($\delta^{18}\text{O}$) for ocean benthic foraminifera and for Devils Hole calcite for the period surrounding Termination II. Note that for the Devils Hole record (solid line) the termination starts much earlier and is more gradual than it is for the ocean record. Also the duration of the interglacial warm interval is twice as great in the Devils Hole record as in the ocean record. Also keep in mind that Devils Hole ages have not been corrected for the aquifer transit time (hence they are minimum ages). If, for example, this transit time is 15,000 years, the chronology would change as shown by the dashed line. For convenience, the sign of the $\delta^{18}\text{O}$ values for the ocean record has been switched. The small arrows indicate the ^{230}Th age measurements constraining the Devils Hole timescale. The arrow at the top is the accepted ^{230}Th age for corals formed during the last interglacial high sea stand, and the shading shows the range of measured ages.

independent timescale which matches perfectly that based on ^{230}Th . Why, after half a million years of uninterrupted growth, should CaCO_3 deposition come to a halt? Perhaps it was related to the unroofing at Devils Hole which provides geologists with access to the fault.

In my estimation the new Devils Hole chronology is more firm than any other available isotopic age in this range. Nowhere else has a high degree of concordance between ^{234}U - ^{238}U and ^{230}Th - ^{234}U ages been achieved. No other archive is better preserved. No other record has so many stratigraphically ordered radiometric ages. Winograd and his coinvestigators contend that their Termination II age is superior because the ages for corals from last high sea stand range from 118,000 to 135,000 years.

One puzzling aspect of the Devils Hole record is the long duration of Termination II. In the ocean record this

change is quite abrupt (see figure). Radiocarbon measurements for the last termination make a very strong case that these changes are very fast and globally synchronous. The onset of the last termination occurred about 14,000 years ago and it was largely complete by about 9,000 years ago. Radiocarbon dating in the Great Basin, where Devils Hole is located, shows that pronounced drying occurred between 12,000 and 13,000 years ago. Lake Lahontan shrank from its maximum size to a series of small isolated remnants⁵ and Searles Lake dried up altogether⁶. So any explanation requiring a 15,000 year offset in the midpoint of Termination II between Devils Hole and the oceans can be firmly rejected. Perhaps the length of the termination in the Devils Hole record can be attributed to mixing in the aquifer. Water enters the aquifer at several places and flows through a complex web of fractures, so that the isotopic message could be spread out. This might explain not only the slow termination but also the overlong warm period that followed it (see figure). But this cannot be so, as the transit time through the aquifer would have to be 15,000 years or more for dispersion to be important. This traverse time would have to be added to the measured calcite ages — giving an even greater mismatch (see figure).

I remain confused. The geochemist in me says that the Devils Hole chronology is the best we have. And the palaeoclimatologist in me says that correlation between the accepted marine chronology and Milankovitch cycles is just too convincing to be put aside.

The one independent check available is radiometric dating of sediments. Although this direct marine dating method should be far preferred to dating of corals, it relies on there being dependable transport of radiogenic ^{230}Th and ^{231}Pa from the water column to the sea floor, and so far only the Caribbean has given a trustworthy site. Nonetheless the date so obtained⁷ for Termination II, 128,000 years, fits the accepted marine chronology and we are back where we started. One side will have to give, and maybe — just to be safe — climate modellers should start preparing themselves for a world without Milankovitch. □

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Tenacious fibres

Atoms attract each other strongly. So why don't solid surfaces stick together on contact? The answer, of course, is that most surfaces are so rough on an atomic scale that they touch at very few points. With more extensive atomic contact, they would stick together firmly.

Nature provides an intriguing example. The gecko, that engaging little tropical lizard, climbs freely up walls and across ceilings, no matter how smooth, rough or porous they may be. It seems to adhere not by glue or suction or mechanical grip but by Van der Waals forces. Its feet have many fine flexible hairs, called setae, which make detailed close contact with any surface. The resulting atomic attractions are strong enough to support the gecko.

Daedalus is now copying this masterpiece of reptilian cunning. His novel 'fractal sticker' is covered with a carefully graded mixture of fibres. The coarsest fibres pack as closely as they can; finer fibres fill the gaps between them; between them are finer fibres still — and so on down to fibres of almost macromolecular dimensions. This subtle structure flows almost like a liquid. Pushed against a surface, the coarse fibres fit themselves into its large-scale contours. The smaller fibres fit round them to fill the smaller irregularities; those smaller still flow into the still tinier gaps, and so on. So the fractal sticker makes intimate contact with any surface, and clings with surprising force. It is best moved by peeling it off from one end, and laying it down in a new position, which is how a gecko moves its feet.

At first Daedalus saw his fractal sticker merely as a novel sole for climbing boots, or a lining for builders' and steeplejacks' gloves. But DREADCO fibre-technologists now plan to make fractal-fibre fabric in bulk, by elaborating the techniques used to make felt, velvet and other furry fabrics. 'Fracfix', as the new product will be called, will cling to any surface without marking or damaging it. It will transform our lives in many ways. Pictures, posters or lettering backed with it will stick firmly on the wall, or even the ceiling, while being easy to remove or reposition. Fracfix-lined belts, straps and webbing will shed their clumsy buckles, and a Fracfix wig will cling immovably to the shiniest bald pate. Fracfix clothing fabrics will give a wonderful hugging fit. They will bring new security to long stockings and strapless gowns, and will make possible even more daringly unsupported creations. And double-sided Fracfix, holding anything to anything and yet easily and repeatedly removed, will be the handyman's dream. David Jones

Correct structure prediction?

SIR — Nearly everyone agrees that one of the 'big' tasks in structural biology is to predict the folded conformation of proteins from the amino-acid sequence. Recently developed prediction methods based on an analysis of aligned identical protein sequences (see ref. 1 for review) have made several predictions that were shown to be remarkably accurate by subsequently determined crystal structures. Does this work represent a "spectacular achievement [that] will come to be recognized as a major breakthrough" (ref. 2)? Or does structure prediction still remain "more a matter for soothsayers than scientists" (ref. 3)?

One need not hold an opinion on this issue to realize that the best way for emerging prediction methods to achieve acceptance is through use. Structures must be predicted and the predictions published *before* crystallographic data are available. This way, knowledge of the structure cannot bias the prediction, the predictions (both correct and incorrect) are visible, and the method is placed 'at risk'. The only obstacle is one of coordination. A prediction published years in advance of a crystal structure is uninteresting. A prediction submitted even days after a crystal structure appears is useless.

We were fortunate that Musacchio *et al.* contacted us a few weeks ago to challenge us to predict the conformation of an SH3 domain (a small domain homologous to various signal-transduction proteins⁴) using prediction methods that we have developed in Zurich⁵. Our prediction has been submitted to the *Journal of Molecular Biology*. Musacchio *et al.* report the crystal structure of an SH3 domain on page 851 of this issue⁶. Thus, readers of *Nature* have another opportunity to compare a crystal structure with a prediction made *de novo*, and can decide for themselves whether there has been a breakthrough, or whether soothsaying prevails.

For those who cannot wait for the paper to appear in *J. molec. Biol.*, we summarize the prediction here. The protein is predicted to be built from β -strands with a single two-turn α -helix lying on one face. The predicted secondary structural elements correspond to the following positions in the sequence of the protein analysed crystallographically (which presumably starts with residues TGKEL): 7–13 (β 1); 19–23 (β 2); 26–33 (α 1); 38–41 (β 3); 48–50 (β 4); and 53–55 (β 5). This prediction contrasts sharply with that made by standard Chou–Fasman and Garnier–Osguthorpe–Robson methods, where the structure is predicted to be largely helical.

Predictions such as these are ex-

tremely important to the development of methods for predicting protein conformation. We welcome additional challenges to make predictions using our method, especially if (1) a structure will shortly be solved; (2) no structure is available for any obviously homologous protein; (3) sequences are sent to us by computer mail together with a few literature citations that provide an overview of the chemistry and biology of the protein family; and (4) this material is sent enough in advance to allow coordination of the publication of the prediction and publication of the structure.

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Speciation events

SIR — Coyne suggests¹ that the adaptive divergence of 'ordinary' genes explains reproductive isolation, in particular Haldane's rule that unisexual sterility or inviability is limited to the heterogametic sex. Others have proposed that 'novel' genetic phenomena make a large contribution to speciation. For example, genomic imprinting and other heritable epigenetic marks² or meiotic drive genes^{3,4} might be involved. If this alternative view is correct then speciation genes, rather than being a representative sample of all genes, will be a distinctive set of genes with particular genetic properties (for example, imprinting, segregation distortion).

In his review, Coyne¹ did not discuss studies of hybrid sterility in the mouse except to note that they support the view that the X chromosome has a disproportionately large effect. In fact, three of the four known mouse hybrid sterility genes are autosomal, and in accordance with Haldane's rule all four genes have male-specific activity. The first known mouse hybrid sterility gene *Hybrid sterility-1* (*Hst-1*) maps to chromosome 17, as does *Hst-4*. *Hst-2* probably maps to chromosome 9 and only *Hst-3* is X-linked. High resolution mapping of *Hst-1*, *Hst-4* and X-linked sterility supports the involvement of 'novel' genetic

events in causing speciation.

Hst-1 has been narrowed down to a region of chromosome 17 that contains at least four interesting genes: *Sod-2*, *tc1-w73*, *Tme* and *Igf2r* (ref. 5). *Sod-2* is a nonimprinted gene which encodes mitochondrial superoxide-dismutase; *tc1-w73* is a recessive lethal factor of a *t*-haplotype. Both the insulin-like growth factor II receptor gene (*Igf2r*) and the T-associated maternal effect gene (*Tme*) are imprinted such that only the maternal copy is expressed in embryos⁶. It had been postulated that *Igf2r* and *Tme* were identical⁷. But when it was found that in interspecies hybrids *Igf2r* and *Tme* assumed different patterns of imprinting from each other⁶ it was concluded that they are probably two separate but tightly linked loci. This interference with imprinting in the hybrid strongly suggests that these loci are involved in speciation.

The *Hst-4* locus causes male sterility in crosses between *Mus domesticus* and *M. spretus*. It, too, is located on chromosome 17 and is not separable from the *t* complex distorter locus *Tcd-2*, which is involved in male transmission ratio distortion⁸. This finding is direct evidence that meiotic drive genes are associated with unisexual hybrid sterility.

X-linked male sterility affects crosses between *M. domesticus* and *M. spretus*. Hybrids show a high frequency of X–Y dissociation in the first meiotic metaphase⁹. In backcrosses, both X–Y dissociation and sterility cosegregate with the *Amel* locus, close to the X–Y pairing region. Genetic divergence of the X–Y pairing region seems to cause X-linked hybrid sterility⁹. Again, this implicates meiotic drive, as lack of pairing between the sex chromosomes is associated with segregation distortion¹⁰. In summary, studies of unisexual sterility in the mouse suggest that the first step towards speciation involves more distinctive genetic events than those suggested by Coyne.

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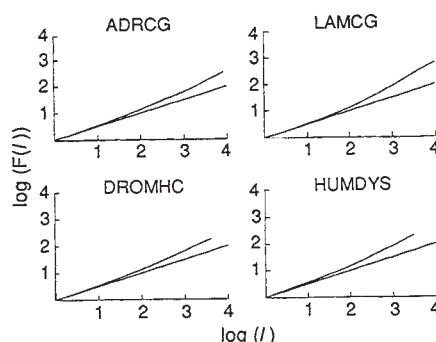
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Correlations in intronless DNA

SIR — Peng *et al.*¹ have reported their finding of long-range correlations in DNA sequences, analysed as purine/pyrimidine 'random' walks. In addition, they suggested that it is possible to distinguish intronless DNA sequences from sequences containing introns using the value of the exponent of a power law calculated on these sequences. They supposed that intronless sequences yield exponents of approximately 0.5 and those containing introns yield exponents greater than 0.5. Nee in Scientific Correspondence² has put forward a different framework for interpreting those results in terms of the already known mosaic structure of genomes.

We tested the algorithm of Peng *et al.* on the 80 largest intronless putative coding regions of yeast chromosome III (ref. 3) with lengths ranging from 1,000 to 6,000 nucleotides. On one strand, 39 such coding regions have been identified; 7 of these open reading frames



The two frames on the left are plots of $\log (F(l))$ against $\log (l)$ for intron-containing sequences reported in ref. 1. The two frames on the right correspond to intronless sequences reported in the same paper. In each frame the continuous curve is obtained if $F(l)$ is calculated as an average over the entire sequence. A straight line of slope $\alpha=0.5$ is included in each plot for comparison.

and those containing introns (see figure). Moreover, in intronless sequences the reported slope of 0.5 appears to be an artefact of the min-max partitioning. The slope is much higher when $F(l)$ is computed over the entire sequence (column 2 of the table). Finally, when the fluctuation is computed as an average over the entire sequence, the slope deviates from 0.5 as soon as the window size exceeds 10–50 nucleotides (see table). This is true for all but one of the 10 intronless sequences reported in ref. 1. The table shows the increase of the slope α with the window size; values appreciably higher than 0.5 are obtained for a window size of 100, representing less than 5% of the length of the smallest intronless sequences used in ref. 1. The figure illustrates the difficulty of discriminating intron-containing sequences from intronless ones on the basis of such a slope analysis.

Whereas (see also ref. 4) the distribution of purine/pyrimidine bases in DNA does not follow a simple random-walk model, the interesting observation of Peng *et al.*¹ cannot be used as a general method to identify intronless sequences.

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yielded exponents between 0.41 and 0.47; 11 had exponents between 0.47 and 0.53; and 21 had exponents between 0.54 and 0.73. On the complementary strand, 41 such coding regions have been identified. Of these, 6 yielded exponents between 0.38 and 0.47; 12 yielded exponents between 0.47 and 0.53; and 23 had exponents between 0.54 and 0.68.

Failing to discern any clear correlation, we decided to verify the algorithm for the same sequences whose exponents were reported by Peng *et al.*¹. The existence of a power law such as $F(l) \sim l^\alpha$ implies that the plot of $\log (F(l))$ against $\log (l)$ is a straight line, the slope of which is α . We found that, in most cases, the plot of $\log (F(l))$ against $\log (l)$, where $F(l)$ is calculated as an average over the entire sequence, is not a straight line. Nonlinear curves were obtained both for intronless sequences

sequences used in ref. 1. The figure illustrates the difficulty of discriminating intron-containing sequences from intronless ones on the basis of such a slope analysis.

Whereas (see also ref. 4) the distribution of purine/pyrimidine bases in DNA does not follow a simple random-walk model, the interesting observation of Peng *et al.*¹ cannot be used as a general method to identify intronless sequences.

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Antibodies from libraries

SIR — We have predicted^{1–3} that combinatorial antibody technology will eventually become the method of choice for the generation of monoclonal antibodies and we stick by that prediction despite the reservations expressed in the Scientific Correspondence by Gherardi and Milstein⁴. In particular, we believe the potential to derive high-affinity human antibody fragments from combinatorial libraries has been clearly demonstrated^{5–8}. Most recently, we have shown recombinant Fab fragments against the HIV-1 surface glycoprotein gp120 or against the respiratory syncytial virus F-glycoprotein to be highly effective in neutralizing the respective viruses⁹. Nevertheless, Gherardi and Milstein raise interesting issues about the scrambling of heavy (H) and light (L) chains inherent in the random combinatorial approach and the likelihood of regenerating the *in vivo* H–L combination ('original antibody' in the nomenclature of Gherardi and Milstein).

Do H and L chains find their original partners in random combinatorial libraries? It has been concluded previously¹⁰ on statistical grounds that they cannot unless one has very large libraries, thus Gherardi and Milstein argue for very complex combinatorial libraries ($>10^8$). However, where it has been looked at, the representation of functional H and L chains in combinatorial immunoglobulin G libraries prepared from immunized animals has been far higher than these theoretical statistical arguments seem to allow. For instance, a study on mice infected with influenza A virus¹¹ found a functional H chain at a frequency of 1 in 50 in a combinatorial library and a functional L chain at a frequency of 1 in 275. The expected frequency of the combination (anti-haemagglutinin) is therefore only 1 in 13,750 and would be present in multiple copies in a library as small as 10^5 . These frequencies are determined by hybridization experiments, which may not distinguish somatic variants of given H and L chains and therefore do not unambiguously describe original H–L combinations according to a strict definition of chains arising from the same B cell. But they do indicate that the diversity of IgG libraries from immunized animals can be restricted and theoretical statistical arguments are fraught with difficulties. In this context, bigger libraries are not necessarily better libraries: library quality in terms of functional binders is dependent on the source of mRNA.

Gherardi and Milstein believe that H and L chains do not find their original

partners based on a comparison of a hybridoma and combinatorial antibody approach to analysis of the murine response to 2-phenyloxazolone (phOx). The predominant H-L combination identified by hybridomas was not found by the library approach. By contrast, the H-L combination identified in the haemagglutinin system described in ref. 11 was one which had been described previously as a contributor to the response from hybridoma studies. We cannot explain these discrepancies, but we repeat the comments of Gherardi and Milstein that the library approach uses mRNA as a source material and therefore should reflect antibodies from plasma cell populations, whereas the hybridoma approach is thought to reflect antibodies from activated but not terminally differentiated B cells. We believe it is inappropriate currently to see either of the approaches as exclusively defining the antibody response.

We agree that H-chain promiscuity (the ability of a single H chain to pair with different L chains with maintenance of antigen affinity) presents formidable problems in attempting to deduce original H-L pairings in the combinatorial approach. In particular, we find that the promiscuity of chain pairing for a protein as antigen⁹ is greater than we had noted for a hapten¹².

As to the question of whether or not it matters if one has the original antibody, we believe in many cases it does not. One exception might be human antibodies intended for use in humans if an unnatural combination were to lead to an immunogenic protein. We feel the risk of this is probably no higher than an anti-idiotypic or allotype response. Another exception might be the study of antibody responses. However, here the available data indicate that the H chains (γ) selected from combinatorial libraries are associated with antigen binding *in vivo* because (1) non-immunized IgG libraries do not generally give the diverse array of high-affinity binders^{5,13} characteristic of immunized libraries; (2)

H chains from immunized libraries appear to dictate specificity when recombined with various unrelated light chains¹⁴; and (3) H chains from immunized libraries can often be grouped into sets of intracloal variants characteristic of an active antibody response (ref 5; C.F.B., manuscript in preparation). Therefore we believe that combinatorial libraries can give much useful information, at least on H chains in immune responses.

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Sizing single DNA molecules

SIR — Sizing of DNA molecules has a central role in virtually every aspect of physical genomic analysis. Although pulsed electrophoresis adequately separates large DNA molecules^{1,2}, accurate sizing remains problematic, and independent size measurements, for comparisons, are often lacking. Here we describe a rapid methodology in which large fluorochrome stained DNA molecules are transiently spread by electrophoresis through a sparse agarose matrix and accurately sized by epifluorescence microscopy, resulting in a highly linear relationship between measured contour length and reported size.

Individual DNA molecules undergoing gel electrophoresis have been previously shown to form hook-like conformations within a tight agarose gel matrix by epifluorescence microscopy^{3,4} and computer modelling studies^{5,6}. Here, we imaged DNA molecules in the gel-coverslip interface⁷, forming greatly elongated hook structures, where molecules electrophoresed unhindered except for interaction with occasional obstacles.

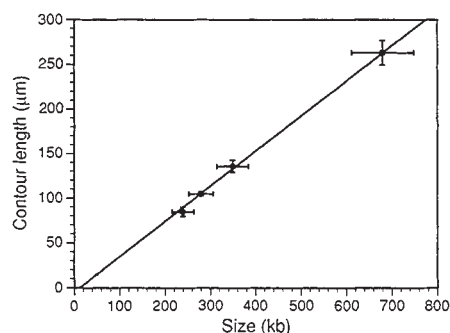


FIG. 1 Correlation of contour length with mapped DNA sizes. Lengths were obtained by digitizing images from a video camera connected to a fluorescence microscope using a 100 $\times$, high numerical aperture objective. Yeast chromosome lengths were derived from mapping data⁹ and pulsed-field gel electrophoresis (PFGE). Horizontal error bars are an estimate of 10% error for PFGE (unpublished results). Symbols reflect (from left to right) averaged measurements, $n=5$, 40, 4, 17, 2, for different DNA molecules with sizes shown in Fig. 2a, b, c, d and e, respectively. Vertical error bars, standard deviation of the measurements. The line contour length (μm) = $0.393 \times \text{size}(\text{kb}) - 4.931$, is a least-squares fit to the averaged data. Detailed methods will be published elsewhere.

Tracking hook formation is an essential part of the optical contour maximization (OCM) methodology. Hooks form in the interface in a few seconds and persist in the extended state for about a second, a sufficient time to collect a few averaged images. The net force at the hook apex is minimized, and a metastable state forms in which the apex is transiently fixed and the two arms can reach equilibrium with the applied field (typically, 30 volts between a 2-cm electrode spacing).

To determine if our conditions produce complete elongation, we used OCM and measured the contour lengths of lambda bacteriophage DNA and four yeast chromosomal DNA molecules. In Fig. 1, the measured contour lengths are plotted against the reported sizes of DNA molecules with a linear least-squares fit, producing an intercept close

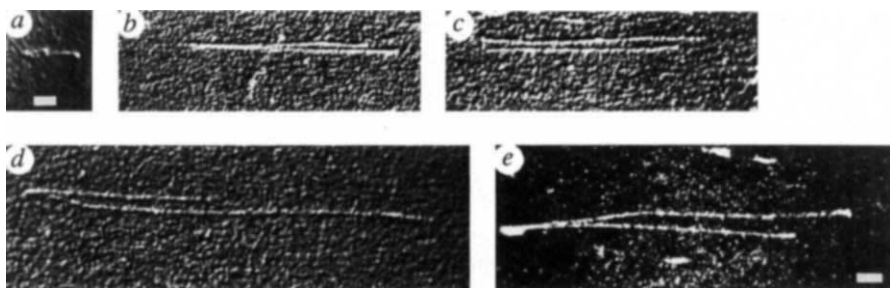


FIG. 2 Gallery of hook images of various DNAs at maximum extension. a, Lambda bacteriophage DNA (48.5 kb). b-e, *Saccharomyces cerevisiae*, AB972 chromosomal DNA molecules⁹, I (245 kb), VI (270 kb), III (350 kb), XI (680 kb), respectively. Bars: a, 5 μm , b-d, same scale as a; e, 10 μm . Digitized, processed images were obtained by epifluorescence microscopy of ethidium bromide-stained DNA molecules embedded in agarose gel.

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to the origin. The excellent linear correspondence found here verifies nearly complete elongation and demonstrates the validity of OCM. Selected images of maximally extended DNA molecules corresponding to the sizes used in the plot are presented in Fig. 2(a-e). Because it is a good approximation to assume that a DNA molecule reaching its most extended state is straight, the apparent contour length is close to the actual contour length. This combination of contour measurement coupled with complete stretching and transient fixation through hook formation forms the physical basis of OCM.

An intercalating fluorochrome, such as ethidium bromide, can unwind a DNA helix and thus increase its contour length⁸. OCM begins to quantify this effect. For the yeast chromosomal DNA molecules, the measured contour lengths are from 4 to 14% longer than the lengths calculated assuming B DNA (0.34 nm per base pair) and using sizes determined by physical mapping⁹. The measured contour length for lambda DNA is 16.5 μm , which matches the length calculated using 48.5 kb. Size discrepancies may also be due to elec-

trophoretic stretching and accompanying distortion of the helix, particularly in larger molecules.

Size determinations of large DNA by OCM are very precise, with an estimated precision of approximately 6%, which is equivalent or superior to most pulsed electrophoresis size determinations. In sum, OCM offers a rapid and precise new methodology for sizing large DNA molecules.

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In defence of radioactive journals

SIR — Those concerned about even minuscule doses of radioactivity will take no comfort in the revelations of Singh and Taylor (*Nature* **356**, 293; 1992) concerning the ionizing radiation emitted by the fine clays used to manufacture the glossy papers used in many magazines and journals. Singh and Taylor calculate that a person standing 0.4 m in front of a seven-shelf bookcase filled with issues of *Nuclear Physics* receives a dose of about 0.6 $\mu\text{rad h}^{-1}$.

Because of the threat that the worst offenders may be banned by zealous regulators, I propose that journal publishers counter by publishing dose rates to which both their readers and regulators are subjected by everyday activities.

Consider, for example, flying aboard a commercial aircraft. For several years I have passed the time flying between cities by recording the background count indicated by a small Geiger counter. On long flights, I use a Geiger counter connected to a palmtop computer. While flying from Pittsburgh to San Antonio last December (see figure), I was subjected to 500 times the dose rate received during my last visit to a library. The dose rate received from a bookcase loaded with radioactive journals is less than the thickness of the trace plotted in the figure. During a recent flight to and from Hawaii, the dose rate was somewhat smaller, but the exposure time was several times longer.

Publishers can appease regulators by suggesting that anxious readers avoid an hour of aircraft travel for every 500 hours spent

in a library. Frequent flyers can relieve their anxiety by declining the magazines passed out by flight attendants. Those who insist on reading while flying should be advised to hold the magazine rather than resting it on their lap. Flyers who are troubled more by boredom than by cosmic rays might take up the entertaining hobby of logging the background count as they fly.

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More on bear droppings

SIR — We note with interest that the polymerase chain reaction (PCR) is increasingly being used to analyse unconventional material^{1,2}. The use of the method to analyse bear droppings reported by Höss *et al.*² marks an advance in the study of wild animal behaviour. We would like to comment on two aspects of their work.

First, we are impressed by the demonstration of plant DNA in the bear droppings. But, given the sensitivity of the PCR method, the amplification of plant genetic materials does not necessarily imply that an animal has fed on plants, but could mean that it has ingested a herbivore that has just fed on grass. This 'Russian doll' effect could theoretically go some way back into the food chain.

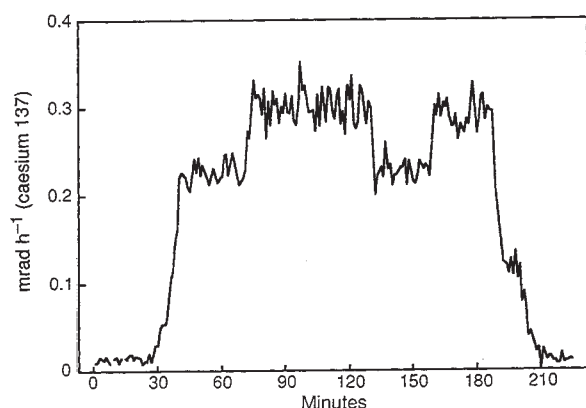
Second, Höss *et al.* demonstrate the potential of applying PCR analysis of excrement to the study of genetic variability and population size. The mitochondrial sequences derived from three Brenta bear droppings (which differed from the American brown bear and the Romanian brown bear samples) reported by Höss *et al.* were identical to each other. Höss *et al.* alluded to the possibility that this is a reflection of the small population size or inbreeding, which may need statistical analysis, as an alternative explanation is that a single bear has deposited droppings in three different places. One way to address this issue is to establish the identity of the individual animals, for example by PCR-based sex determination or DNA fingerprinting. This information could allow the movements of individual animals to be followed by tracking the droppings.

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Background radiation received aboard a commercial aircraft flying from Pittsburgh, Pennsylvania, to San Antonio, Texas. The two highest peaks correspond to an altitude of 10,670 m; the two secondary peaks to an altitude of 9,450 m. The cluster at 0.1 mrad h^{-1} near 195 min resembles what is typically observed when an aircraft levels off during its descent. (Instrument: RadAlert (International Medcom, 7497 Kenney Road, Sebastopol, California 95472).)

Chemistry in context

Peter Harman

Ideas in Chemistry: A History of the Science. By David Knight. *Athlone*: 1992. Pp. 213. £38.

Science as Public Culture: Chemistry and Enlightenment in Britain, 1760–1820. By Jan Golinski. *Cambridge University Press*: 1992. Pp. 342. £32.50, \$54.95.

From Caveman to Chemist. By Hugh W. Salzberg. *American Chemical Society/Royal Society of Chemistry*: 1991. Pp. 294. \$24.95, £18 (hbk); \$14.95, £11 (pbk).

In the introductory 'biography of chemistry' in his *Ideas in Chemistry*, David Knight distinguishes the concerns of chemists' histories from the range of problems conceived by modern historians of chemistry. Whereas the former are sure of their subject as a progressive scientific enterprise, the latter — and this is especially true of the most recent historical work in the field — have come to find the definition of chemistry and its history as problematic. Knight himself inverts the traditional assumption of triumphant chemical progress, preferring to regard chemistry as "having a glorious future behind it".

From the questioning perspective of modern historical writing therefore, Hugh Salzberg's *From Caveman to Chemist*, though worthy in execution, has an antique tone. This is the confident tale of chemical progress with the beginnings of true chemistry found in the work of Boyle and van Helmont in the seventeenth century. From this perspective, the phlogiston theory of the existence of a hypothetical substance in all combustible materials is "strange" but nevertheless "useful" to Priestley and others who made "first-class discoveries". This prepares the ground for the chemical revolution of Lavoisier and a brief treatment of nineteenth-century atomism and organic chemistry. The bulk of the book is given over to ancient technology, alchemy and the origins of chemistry, drawing on the standard sources.

It is to avoid the difficulties inherent in such an account of the history of chemistry that Knight seeks to investigate the roles chemistry has played during its history. He sees the characteristics of chemistry as changing over time, and suggests that its history should be investigated by study of the ideas external to the science that have shaped its development and character. His book is structured by a treatment of chemistry under such headings as "Mechanical", "Fundamental", "Experimental" and "Useful"; and his narrative is only loosely chronological. Knight's point of view is very suggestive, but it is unfortunate that his perspective on chemical history remains programmatic. He writes within a brief compass, and his style is

discursive and informal, lacking rigour and systematic development of the material. The general scientific reader, who may be attracted by the book's informal style, will be disappointed to find a book on a subject so replete with intriguing apparatus, handsome laboratories, evocative symbols and suggestive diagrams to be utterly bare of any form of illustration. Knight's essays would have been better served if accompanied by illustrative material chosen to complement and illuminate the argument.

Jan Golinski's substantial monograph on *Science as Public Culture* offers an original approach to the writing of chemical history. Its subject matter is the history of chemistry in Britain during what must be its most exciting period, that embracing the careers of William Cullen, Joseph Black, Joseph Priestley and Humphry Davy. Golinski is not overly concerned with the chemical theories of these men (which never receive detailed exposition here), but instead concentrates on the way these chemists functioned within the public culture of the day. As the title of his book makes clear, Golinski is concerned with the relationship between scientific work and its social and political context. As Knight observes, sociological approaches such as Golinski's "can lead to the history of science with the science left out", and Golinski largely bypasses the traditional concerns of the history of chemistry. But there are compensations: in such an account as Golinski's, phlogiston is not a conceptual oddity and continued adherence to it a mystery, but is seen as embodied in a matrix of scientific practice and rhetoric.

Golinski has written a compelling account of this seminal period of chemical history. This was indeed the heyday of chemistry, both in its public impact and as the period of the 'chemical revolution', in which the innovations of Lavoisier transformed the science of chemistry. The complex relationship between the British chemists and their French colleagues forms one of the main topics addressed here. The acceptance or rejection of Lavoisier's new chemistry is not to be understood in terms of competing theories of combustion and the rationale underlying them, but as elements in a complex debate about how science should be practised and established publicly. Golinski places special emphasis therefore on the value systems with which different systems of science were associated.

In Britain, the 1790s witnessed a strong reaction to the values of the Enlightenment. Priestley's programme of chemistry as the vehicle of social progress was savaged and ridiculed in the chaos surrounding Thomas Beddoes' discovery of the intoxicating effects of



Photo opportunities — cartoon of the working photographer on the cover of the *New York Daily Graphic* (30 December 1875). The editors were enthusiastic about using photographs: "We propose to illustrate daily occurrences in such a way that the life of our times shall become photographic; and the illustration of events will be as accurate and pleasing and elegant as any word painting in the text". This picture is taken from *The Origins of Photojournalism in America* by M. L. Carlebach (Smithsonian Institution Press, \$29.95 (pbk)).

nitrous oxide. Davy, freeing himself from Beddoes, who was his patron, carved out a metropolitan career as lecturer and scientific genius, articulating a conservative version of chemistry serving a stable and stratified society. The Priestleian invocation of public participation in scientific demonstration was replaced by awe for the specialist surrounded by his instruments. Here Davy adopted the rhetorical and technical practices of Lavoisier, so castigated by Priestley.

Golinski sees the chemistry of the period intimately bound up with the fortunes of enlightened values and forms of civic life, not as conditioning factors but as intrinsic to the development of chemistry as a science. The concerns of his study are directly congruent with

those of social and cultural historians — to chart the role of intellectual life in public culture. As Knight observes, for all the lip service paid to the 'age of science', historians have remained largely impervious to the historical impact of science. It is to be hoped that Golinski's study will help to establish the central importance of science in the mainstream of historical study. □

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■ The newly launched Fontana History of Science Series, edited by Roy Porter, aims to provide a comprehensive synthesis for historians, scientists and nonspecialists. The first two titles on chemistry (by W. H. Brock; received too late for inclusion here) and the environmental sciences (by P. J. Bowler) will be reviewed in a future issue of *Nature*.

The promiscuous primate

Adrienne Zihlman

The Last Ape: Pygmy Chimpanzee Behavior and Ecology. By Takayoshi Kano. Stanford University Press: 1992. Pp. 248. \$45.

PYGMY chimpanzees (*Pan paniscus*) differ from the better known species (*Pan troglodytes*) in walking more frequently on two feet, having sex more often and being less aggressive and more friendly. Except for the pelvis, the *P. paniscus* skeleton shows a striking resemblance to fossils of the earliest hominid, *Australopithecus*. So *P. paniscus* provides an alternative and in some ways more congenial model than *P. troglodytes* for the common ancestor of apes and humans, a model much debated during the past decade.

Contributing to this debate, Takayoshi Kano's delightful book summarizes his more than 10 years of field research on pygmy chimpanzees. Although published in 1985 in Japanese "for lay people interested in animals", it remains a useful overview of chimpanzee ecology and behaviour. Evelyn Ono Vineberg has done primatology a service with her clear translation of this succinct and informative volume.

When Kano began his initial survey of the forests of the Zaïre River Basin in 1973, nothing was known about the free-ranging population of pygmy chimpanzees. In the best tradition of Japanese long-term primate studies, Kano and colleagues have since been carefully accumulating observations of this elusive species. They have learned that pygmy chimpanzees are unusual among primates in that males, not females, maintain lifelong closeness to their mothers, and Kano believes that the lack of both

aggression and male bonding is due to this behaviour. Males seem to obey the 1960s' injunction 'Make love, not war', for they copulate frequently, as often as 11 times in one day.

Copulation in tense situations may, Kano suggests, play a social role in "preventing antagonism and facilitating peaceful coexistence between males and females". The front-to-front copulation position, thought to be characteristic of the species, turns out to be more popular among adolescent males and females than among fully grown adults, although adult females "clearly prefer the ventral position".

Females maintain close ties with each other and express this friendship by rubbing their genitals together. Adolescent females move from group to group,

but eventually stay in one group and, after giving birth, become stable members. "This reminds me of a new Japanese bride", Kano remarks, "who bravely enters the groom's large household. If she marries the oldest son, she may also have to live with her husband's parents and the unmarried brothers and sisters of her husband. Because she is picked on by in-laws, and her own family is distant, the new bride is often under great stress." Nevertheless, *P. paniscus* society is much less hierarchical than that of *P. troglodytes* and males do not show the same dominance over females.

Although it has become popular to use the term 'bonobo' for *P. paniscus*, Kano sticks to the original name 'pygmy chimpanzee', which emphasizes the demonstrated close genetic relationship between the two species. 'Bonobo' was introduced into the literature in 1954 by E. Tratz and M. Heck as a native name for the animal, but during his work throughout the region, Kano never heard this word spoken by local people. Its use in the current literature has taken on some political overtones, sometimes implying that the animal is not a true chimpanzee or perhaps not a chimpanzee at all.

Japanese primatologists have traditionally provisioned their study groups, including those of pygmy chimpanzees, a controversial practice said to alter the nutritional state, social interactions and organization of group members. This criticism has also at times served to imply that a few hours of observation of a few animals by researchers who do not provision is somehow more authentic than the years of patient and persistent work done by Japanese primatologists. Kano estimates that pygmy chimpanzees depend on provisioning for about 1.5 months every year. But without provisioning, there would be few observations to report, owing to the density of the forest habitat. Like so much in life and research, there is a trade-off.

An invaluable feature of the book is the detailed comparison of *P. paniscus* behaviour with that of *P. troglodytes*. What emerges is a comprehensive picture of the genus *Pan*. The book therefore provides an excellent foundation for integrating from continuing studies new data presented in the primary literature.

It is remarkable that so much information could be so pleasantly and efficiently enclosed in a mere 248 pages, including 12 figures, 8 maps, 26 tables and 78 photographs — all tied together with a relaxed and lucid prose style. The book, like its subject, is a model that anthropologists will study with great interest. □

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A pygmy chimpanzee with young.

Don't steal this book

Jeffrey Mervis

Stealing into Print: Fraud, Plagiarism, and Misconduct in Scientific Publishing. By Marcel LaFollette. *University of California Press*: 1992. Pp. 298. \$30.

THE modern era of scientific misconduct began in 1974, when William Summerlin, a dermatologist, used a felt-tip pen on two mice that had received skin grafts to create a result that could not be achieved experimentally. Since then, all sorts of questions have been raised about the prevalence of misconduct, its origins and its impact on society, and much has been said about how the scientific community can prevent it or, failing that, respond quickly and effectively.

Marcel LaFollette does an admirable job narrating these debates, pointing out their high and low points and commenting on their importance. What she does not do is suggest any answers or provide a roadmap to the next stage. Instead, the reader is offered innocuous advice on how misconduct should be handled, with each player in the drama being told to act wisely and responsibly.

Such restraint need not be the case. LaFollette has followed most of the incidents of the past dozen years that have captured headlines, filled congressional hearing rooms and generated an untold number of panel discussions. As the editor of *Science, Technology and Human Values*, she was briefly a participant in one notorious episode — the attempt by Ned Feder and Walter Stewart, two scientists at the US National Institutes of Health, to publish their investigation into the co-workers of John Darsee, a cardiology researcher who fabricated data in dozens of papers.

There is no question that LaFollette knows her stuff, and she is not afraid to scrutinize the actors. She correctly scolds the scientific press for being too trusting, calling them “drowsy watchdogs rather than hyperactive pit bulls” in uncovering misconduct, and she captures the smugness among those who have followed the issue for years by describing the “knowing glances” exchanged by those “trying pretentiously to look like insiders” at public gatherings.

Regrettably, the author ignores her own observation, offered about scientists as a whole, that “they apparently do not learn political lessons as easily as they learn new lab techniques”. Government bodies are being forced to deal with scientific misconduct because the scientific community — journal editors,



The coyote *Canis latrans* from Audubon's *Viviparous Quadrupeds of North America* (1845–46). The picture is reproduced here from P. T. Stroud's *Thomas Say* (University of Pennsylvania Press, \$24.95), a biography of the pioneering New World naturalist who gave the animal its scientific name.

laboratory directors, peer reviewers, professional societies, university administrators and so on — have repeatedly made a mess of things. Politicians, even the US Representative John Dingell (Democrat, Michigan), focus on the occasional instance of misconduct not out of meanness towards science but because they take seriously their stewardship of public funds, of whatever amount.

What US legislators are waiting to see is evidence that the scientific community is capable of rooting out and dealing effectively with misconduct. Once assured of that, they can turn their attention back to the likes of savings and loan scandals and defence overbillings. LaFollette would have done better to point out reasonable, or at least provocative, solutions. Instead, she offers such bromides as “Where does the responsibility for thorough evaluation [of manuscripts] lie? An obvious response is ‘everywhere’”. The questions that she raises at the outset, including whether authors, referees and editors are sufficiently accountable, whether the literature can be trusted if peer review cannot detect fraud and whether the data are sufficiently accurate to be used for public policy decisions, are important, but they go unanswered.

It seems likely that the attention paid to misconduct will wane once scientists show an ability to deal with the problem. But it seems unlikely that *Stealing into Print* will help them much in achieving this goal. □

Jeffrey Mervis is news editor of *Nature*.

People and places

A. M. Mannion

Late Quaternary Environmental Change: Physical and Human Perspectives. By M. Bell and M. J. C. Walker. *Longman*: 1992. Pp. 273. £15.99, \$39.95 (pbk).

IT is encouraging to note that at a time when many texts are preoccupied with current and possible future environmental change, more than lip-service is being paid to past environmental change. Such is the main focus of this well written and well produced book.

Although a wide range of topics is covered, including many almost hackneyed themes such as the greenhouse effect and ozone depletion, there remains a commendable emphasis on scientific method: scientific rigour is all too often lost amid the overwhelming volume of issue-based literature; and students often shy away from theory and philosophy. Also examined are the evidence (fossil, sedimentary, recorded and so on) for environmental change and the usefulness of the dating techniques used to cover the past 20,000 years, which provide the temporal setting for the book. There is, however, little overt criticism of these methods or much discussion of their limitations.

The subsequent narrative on natural environmental change, particularly climatic change, makes interesting read-

ing, detailing not only the causes but also the consequences of change for both landscape and people. However, the influence of environmental change on some human activities, such as the origins of settled agriculture, is not made at all clear. The chapters that detail the effects of people on the environment provide useful synopses concentrating on erosion, which is examined on a regional basis, and climate. The chapter on conserving the cultural landscape falls, somewhat uncomfortably, between the topics of erosion and climate but its subject matter is both valuable and novel. Once again, the complex and inalienable components of nature and culture are apparent, although to the book's detriment there are no suggestions for possible frameworks in which to examine the relationships between people and the environment. The same is true of the all-too-brief postscript. This, for good or ill, manages to avoid discussion of the, albeit poorly-defined, concept of sustainable development.

Overall, the book presents a worthwhile synthesis of the history of people and the environment. It is a timely reminder that the character of this relationship is about to change: the question is whether this change will be by design or by default. □

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■ Key issues relating to debates on environmental change and policy are also examined in the new introductory text *Environmental Issues in the 1990s* edited by A. M. Mannion and S. R. Bowlby. Published by Wiley, £14.95 (pbk).

Degrees of uncertainty

E. H. Mamdani

Fuzzy Systems Theory and Its Applications. By Toshiro Terano, Kiyoji Asai and Michio Sugeno. *Academic*: 1992. Pp. 268. £33, \$49.95.

VARIABLES applicable to such areas as expert systems, knowledge engineering and artificial intelligence often cannot be specified with exact numerical values, as only vague information about them is available. Fuzzy logic is a method for handling such inexact information by attempting to quantify value judgements in such a way that they are amenable to processing by a computer. Often this amounts to creating a fuzzy (or 'fuzzied') version of the conventional theory; the method incorporates rules for manipulating fuzzy sets, which are sets of values corresponding to a fuzzy proposition. Fuzzy set theory has already had practical applications, for example in regression analysis, mathematical programming, diagnosis and systems control. This book provides a good overview of fuzzy set theory, with separate chapters devoted to the research results in each of these areas.

Fuzzy set theory was first introduced by L. A. Zadeh in 1965; the first book on the subject, A. Kaufman's *An Introduction to the Theory of Fuzzy Subsets* (Academic), was published ten years later. Since then, many other books have appeared in the field, but they have mostly dealt with only research results. Only H. J. Zimmerman's *Fuzzy Set Theory and Its Applications* (Kluwer-Nijhoff, 1985) had any real appeal as a

textbook. So it is pleasing now to have this new introduction, which, although primarily aimed at Japanese universities, can also be used elsewhere. The book was originally published in 1987 in Japanese. The English translation is excellent and very readable, although its Japanese origins are still plain to see.

Fuzzy set theory has been developed by workers all around the world, but the theory has never had wide appeal to mainstream researchers in Europe and the United States. Only Japanese (and perhaps also Chinese) workers have so far felt that the theory addresses an important need to represent vague information and deal with it scientifically. The authors of this new book, who are eminent teachers and research workers in the field, may not convince many Western researchers of the theory's value, although they do provide an insight into the kind of contributions that Japanese workers see it making to computer-based applications. □

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Autumn Books

Nature's next review supplement is Autumn Books, which is published on 26 November. The issue is due to feature, among others, Ed Regis on Arthur C. Clarke, Igor Aleksander on *The Turing Option* and Martin Gardner on spontaneous combustion.

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Dipolar reversal states of the geomagnetic field and core–mantle dynamics

Kenneth A. Hoffman

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Palaeomagnetic records from lavas suggest that at least two specific inclined dipolar field configurations have dominated the reversal process for the past ten million years. These long-lived states provide a way to explain directional rebounds, aborted reversals and the recording in sediments of what appear to be preferred longitudinal paths of the virtual geomagnetic pole. The polar orientations correlate with near-radial flux concentrations recognizable when today's field is stripped of its axial dipole, and with lower-mantle seismic anomalies, suggesting a tie to deep-Earth dynamics.

IT is through the palaeomagnetic record preserved in rocks that we obtain the most direct information on the behaviour of Earth's magnetic field during polarity reversal. Most of these records have been obtained from sedimentary structures, and many of the remainder arise from stacked sequences of lavas. These two rock types acquire magnetic remanence in different ways and their records often show distinct palaeomagnetic characteristics¹. In particular, in transitional field data derived from continuously deposited sediments there is a greater tendency for the magnetic vector to rotate in a plane throughout a reversal than there is in records derived from lavas. The degree of directional planar rotation is positively correlated with the degree of longitudinal confinement of the corresponding path of the virtual geomagnetic pole (VGP)².

It has been suggested that not only are reversal paths of the VGP revealed in sediment records longitudinally confined, but that they preferentially lie within two particular, rather narrow antipodal bands, one passing through the Americas and the other through eastern Asia and western Australia^{3–5}. Although the statistical significance of this observation has been questioned⁶, attention has been redirected to dipole-dominated rather than non-dipole-dominated solutions to transitional fields^{5,7,8}. It can be shown, for example, that if the axial dipole alone were to reverse by decaying through zero today, then VGP reversal paths would statistically be biased over the Americas⁸. This result simply illustrates the control that the orientation of the equatorial dipole term would have during such a process.

In reversal data obtained from lavas⁹, however, I found as a common feature not preferential longitudinal confinement of the path of the VGP, but rather two localized regions in the Southern Hemisphere defined by clusters of sequential VGPs contained in a number of records. Furthermore, the two regions, one near South America and the other near western Australia, mainly lay within the longitudinal bands deduced from sediment data.

Although it is always possible in lavas for a tight sequential grouping of recorded palaeodirections to be the result of a short episode of rapid-fire volcanic activity, the similarity of VGP cluster locations among records of different transitions suggested the existence of particular recurring transitional field states which dominate the process of geomagnetic reversal. But my earlier study⁹ considered only Plio-Pleistocene reversal records from lavas of the Hawaii and Society Islands hotspots. These data supported the contention that reversing fields assume similar long-lived configurations, but were not enough to distinguish whether one or two such field states might be involved in the process. Moreover, the dipolarity of each possible configuration was not resolvable given the limitations of the dataset⁹.

Here I consider an expanded lava-derived dataset comprising records obtained from five sites scattered over the Earth.

I will show that these data, nearly spanning the past 10 Myr of geomagnetic reversal history, strengthen the case for recurrence and domination of the reversal process by at least two particular transitional field states, each characterized by a virtually invariant inclined dipolar geometry. There is little evidence in the lava data for continuous longitudinal movement of the VGP.

I will also show that geomagnetic features which for the most part are associated with today's non-dipole field correlate well with the dipole orientation of each hypothesized transitional field state. This correspondence suggests that sources responsible for field complexity during times of full polarity may individually produce inclined dipolar states during field reversal. Finally, I

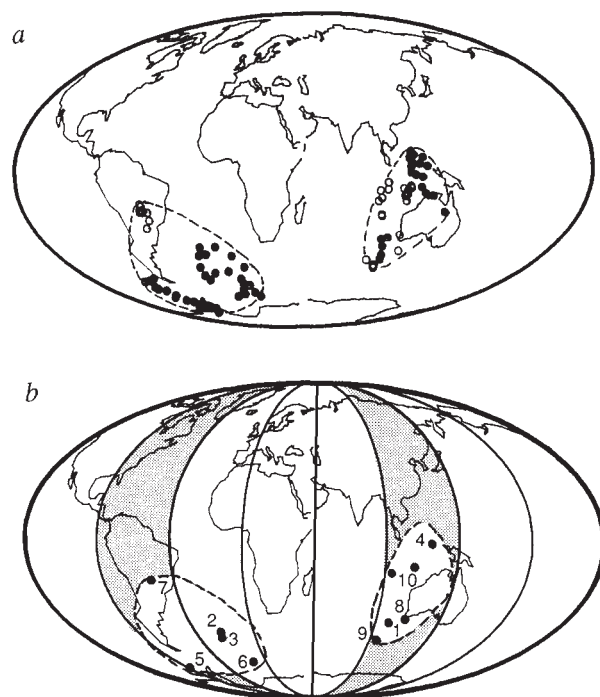


FIG. 1 *a*, Clustered virtual geomagnetic poles (VGPs) contained in six single-cluster records (●; see Table 1) and four more-complex records (○; see text) forming the indicated dual-patch distribution. These data were obtained from late-Cenozoic lavas from five widely scattered sites about the globe. These transitional data strongly suggest that reversing fields are dominated by particular long-lived, inclined dipolar field states. *b*, VGP cluster means corresponding to the 10 datasets (reference numbers refer to those in Table 1 and the text). The cluster patches are found within the indicated longitudinal bands claimed to be preferred by VGP paths obtained from sediments⁵.

will show that anomalies in seismic wave speed in the lower mantle correlate well with both the palaeomagnetic data and the geomagnetic features, providing a possible link to long-term dynamics of the Earth's deep interior.

Lava-derived VGP clusters

Interpretation of palaeomagnetic reversal records obtained from lava sequences is complicated by the lack of knowledge of the absolute time between successive eruptions: a few eruptions over a period of weeks or months could give a cluster of similar directions that has no relevance to long-lived transitional states of the field. For this reason I first construct a dataset containing only those lava records of completed reversals which are dominated by a single tight cluster of chronological palaeodirections.

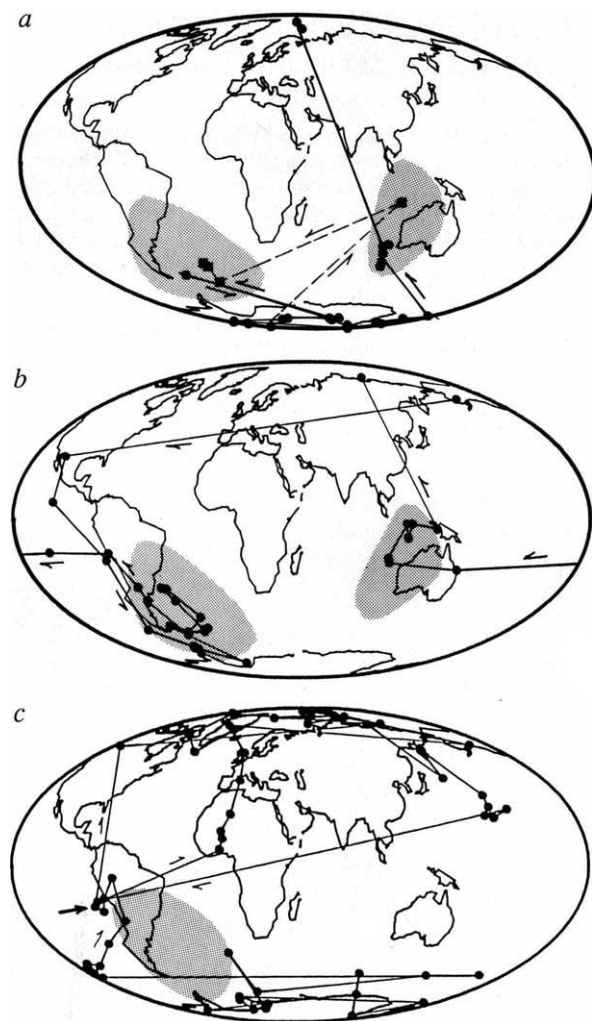


FIG. 2 *a*, Transitional virtual geomagnetic polar movement between the two cluster patches defined in Fig. 1*a*, observed in palaeomagnetic recordings from lavas erupted at the Society Islands hotspot (17° S 209° E), one from near the time of the Olduvai-Matuyama N-R reversal exposed on Moorea (■) (under investigation at present), and the other during the Matuyama-Brunhes R-N reversal exposed on Tahiti¹¹ (●). These transitional data provide further support for the contention that the two VGP cluster patches correspond to two distinct inclined dipolar field states. *b*, From a record of an unsuccessful attempt by the field to reverse in the late Brunhes. The data come from an investigation¹⁷ of Mediterranean sediments (32° N 34° E). The recorded VGPs are first observed within the South America/South Atlantic cluster patch and then after an apparently rapid swing are found in the western Australia cluster patch. *c*, VGPs associated with the Miocene R-N reversal obtained from lavas from Steens Mountain, Oregon¹⁹. Note that the recorded directional rebound (indicated) involves VGPs near the western periphery of the South America/South Atlantic cluster patch.

Specifically, I require that the VGP cluster should be associated with five or more palaeodirections having a 95% confidence cone about their mean $\alpha_{95} < 10^\circ$ and comprise more than 50% of the recorded transitional VGPs. By applying these criteria to all available lava-derived data I ensure an unbiased 'single-cluster' dataset. I am aware of five published reversal records that satisfy these conditions: data obtained from volcanic sequences on New Zealand, Iceland, Tahiti and two islands formed at the Hawaii hotspot (see Table 1).

The dominating VGP cluster associated with each of these five single-cluster records is shown in Fig. 1*a*. Also shown in Fig. 1*a* is the VGP cluster associated with a sixth record, early results from my continuing study of a Pleistocene normal-to-reverse (N-R) transition recorded on Reunion Island (see Table 1). This record, satisfying the single-cluster criteria, contains a dominant VGP cluster in the South Atlantic. The VGP cluster mean associated with these Reunion Island data is virtually identical to that associated with a more recent Pleistocene record from Molokai, Hawaii⁹ and, moreover, is close to that of the late-Miocene record from New Zealand (Fig. 1*b*; Table 1). Clearly, available single-cluster reversal records display not a random distribution of clustered VGPs, but rather two regions of concentration, one near western Australia and the other incorporating the southern portion of South America and the South Atlantic.

In addition to the three single-cluster datasets obtained from the Hawaii and Society Island hotspots (Table 1, Fig. 1), four additional detailed records from these sites were considered in ref. 9. Although too complex to be categorized as single-cluster recordings, each dataset contains a VGP cluster near one of the two regions described above. The Matuyama-Brunhes reversal record (no. 7) from Maui, Hawaii, contains a sequence of about 22 flows having similar palaeodirections which correspond to a tight cluster of VGPs in southeastern South America¹⁰. The records of the termination of the Jaramillo subchron and the Cobb Mountain event from Tahiti, Society Islands (nos 8, 9) each contain sequential VGPs found off the southwestern coast of Australia¹¹ and close to the single cluster of Matuyama-Brunhes transitional VGPs obtained from the same site. Finally, the complex Pliocene reversal record (no. 10) from Huahine, Society Islands, contains palaeodirectional groupings extensively recorded and instrumental in the construction of a composite dataset from individual stacks of lavas¹². The corresponding VGPs cluster near northeastern Australia.

These VGP clusters are plotted with open circles in Fig. 1*a*. Now containing clustered VGPs associated with 10 late-Cenozoic reversal records obtained from five widely separated sites around the world, the composite dataset (Fig. 1*a*) is seen to define a rather remarkable Southern Hemisphere dual-support distribution. I suggest that this finding provides strong support not only for the contention that the reversal process is dominated by long-lived and recurring transitional field states⁹, but also that the two virtual pole cluster localities are in fact associated with standing transitional orientations of the true geomagnetic pole during field reversal. That is, transitional fields are themselves characterized by particular inclined dipolar states.

Any such quasi-stationarity of the reversing field statistically would be expected to result in recorded palaeodirectional clusters in lava sequences and recorded standstills in sediment records possessing a high degree of fidelity. For those sediments in which remanence acquisition involves considerable time-averaging, however, any long-lived transitional field state would cause a directional bias to the palaeomagnetic record. For the case of a single directional standstill during a change in polarity, some degree of spurious longitudinal confinement of the path of the VGP would be expected to pass near the actual stationary transitional geomagnetic pole^{9,13}. In fact, the two VGP cluster patches deduced from lavas are largely contained in the two antipodal longitude bands observed in the composite sediment data³⁻⁵ (Fig. 1*b*).

TABLE 1 Single-cluster records

No. reversal	Age	Sense	Site	Coordinates	N	Declination (°)	Inclination (°)	α_{95}	n	Reference
1 Matuyama-Brunhes	0.7	R-N	S	18° S 209° E	5	229.8	1.5	5.1	1	11
2 Olduvai-Matuyama	1.6	N-R	H	19° N 205° E	5	134.7	-63.5	5.1	0	9
3 Reunion-Matuyama	2.1	N-R	R	21° S 56° E	7	226.3	42.1	8.6	3	*
4 Pliocene 'R3-N3'		R-N	I	65° N 339° E	16	47.1	-33.1	7.1	1	35
5 Pliocene	4.5	R-N	H	19° N 205° E	20	154.9	-22.0	2.9	2	18
6 Miocene	8.5	R-N	NZ	44° S 173° E	7	180.7	27.0	4.1	0	15

N, number of flows contained in directional cluster; n, number of other transitional directions contained in record. H, Hawaii hotspot; I, Iceland; NZ, New Zealand; R, Reunion Island; S, Society Islands hotspot.

* This study.

Inclined dipolar states and their recurrence

The claim that standing transitional field states are dominated by an inclined dipolar geometry assumes that each of the two VGP patches in Fig. 1 is associated with a distinct field state. Considerable support for this is given by early results (Fig. 2a) from a newly acquired partial transition record obtained from lavas on Moorea (extruded at the Society Islands hotspot). This record, probably corresponding to transitional field behaviour near the termination of the Olduvai subchron¹⁴, contains first a transitional VGP within the western Australia cluster patch and then a series of three VGPs within the other cluster patch in the South Atlantic. The simplest interpretation of these Moorean reversal data is that the sampled section of lavas records instants before and after a transformation in field geometry from one inclined dipolar state to the other.

In like fashion, movement of the VGP from one cluster patch to the other, this time in reverse order to the Moorean data, is observed in the Matuyama-Brunhes reversal record obtained from Tahitian lavas (Fig. 2a). Specifically, a time in which the pole resided in the South America/South Atlantic VGP cluster patch precedes an apparent long stand by the pole in the western Australia VGP cluster patch. With no other transitional directions recorded, the polarity transition possibly then completes rapidly.

The single-cluster dataset from lavas (Fig. 1a, Table 1) contains transitional behaviour primarily during the Plio-Pleistocene. The one exception is the record of a Miocene reversal from Akaroa, New Zealand¹⁵. For this case, recorded field behaviour consists exclusively of a single cluster of transitional VGPs. This cluster is found in the South Atlantic in close proximity to the transition data from Reunion, Molokai, and Moorea. The striking similarity among these results from the Miocene through the Pleistocene suggests that the minimum time constant for the occurrence of a particular transitional dipolar field configuration is $\sim 10^7$ years. Such a duration is

compatible with the timescale for mantle convection¹⁶, supporting the claim that inhomogeneities related to the core-mantle system control the orientation of reversing fields⁵.

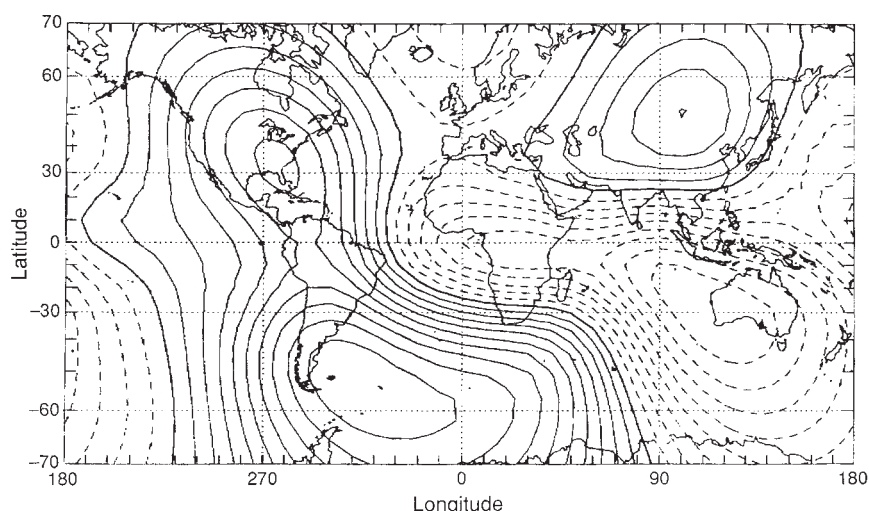
If the geomagnetic field has been dominated by similar inclined dipolar states during reversals from the Miocene through the Matuyama-Brunhes (the last reversal), then it is possible that the same core-mantle conditions have persisted through the Brunhes normal epoch. A record of a late-Brunhes geomagnetic excursion, possibly an aborted reversal, obtained from exposed Mediterranean sediments¹⁷, provides evidence that this may indeed be the case. The path of the VGP (Fig. 2b) is seen first to move into the South America/South Atlantic cluster patch and remain there for a considerable time. Then, with apparent rapidity, it swings directly to the northwest coast of Australia and resides for a time within the other cluster patch. Following this, normal polarity is restored.

This sediment-derived dataset adds credibility to my interpretation of the composite lava-derived findings (Fig. 1), supporting both the existence of two recurring inclined dipolar field states during geomagnetic reversal and their domination of the process. The record further suggests that conditions of the core-mantle system responsible for these hypothesized transitional dipolar orientations have persisted throughout the Brunhes, possibly to the present day. I will return to this possibility later.

Stop-and-go, rebounds and aborted reversals

Observations from lavas and sediments alike have indicated that directional changes during polarity reversal can occur in short bursts ('stop-and-go' behaviour)^{15,18,19-22}. There is now evidence that during the 'go' phase, directional motion may become astonishingly rapid²³. On occasion, palaeomagnetic reversal records indicate an apparently rapid return to a remarkably similar transitional direction. Of such directional 'rebounds'²⁴, the most famous case is that recorded in the thick sequence of Miocene lavas at Steens Mountain, Oregon¹⁹ after an initially

FIG. 3 Contours of the vertical component of the 1975 International Geomagnetic Reference Field at the Earth's surface for the case in which the axial dipole term is removed. Downwardly directed and upwardly directed field are denoted by solid and dashed contour lines, respectively. Note the correspondence of the two Southern Hemisphere features with the VGP cluster patches (Fig. 1a).



unsuccessful attempt by the field to reverse fully. The VGP path for this R–N reversal is shown in Fig. 2c. Although other clusters are contained in the record, the locality of the remarkable rebound is close to the western perimeter of the South America/South Atlantic VGP cluster patch defined by more youthful lavas (Fig. 1a).

Figures 1 and 2 provide strong support for the contention that stop-and-go directional changes are a primary characteristic of geomagnetic field reversal. These data also suggest that directional rapidity may occur during major transformations in field structure between axially dipolar (full polarity) states and inclined dipolar (transitional) states. Behaviour of the VGP during some excursions (aborted reversals) and directional rebounds (Fig. 2b, c) further suggests that these phenomena are an extension of the same dynamo processes responsible for successful reversals²⁵.

Comparison with today's geomagnetic field

It has been suggested that present characteristics of the geodynamo may provide clues to understanding reversal data. Clement²⁶ recognized that the longitudinal distribution of transitional VGPs from sediment-derived data resembled observable features in the downward-continued core–mantle boundary field²⁷. Laj *et al.*⁵ have further noted that this antipodal VGP banding also correlates with the locations of deduced north–south fluid flow at the top of the core²⁸. The recorded transitional VGP clustering in lavas, showing little evidence of longitudinal path confinement, prompts us now to ask whether the present field provides any clues about the process by which particular inclined dipolar states may develop during polarity reversal.

Features of downwardly directed flux are observed in the core–mantle boundary field²⁷ below areas of the South Atlantic and, in particular, near South America; the Southern Hemisphere is otherwise dominated by upwardly directed flux. Given that these flux patches seem to have strengthened over historic time in a manner consistent with progressive weakening of the geocentric axial dipole²⁹, it has been proposed³⁰ that continued growth may ultimately cause polarity reversal. Such a mechanism is not inconsistent with the palaeomagnetic observation of a transitional VGP cluster patch in the South America/South Atlantic. But these core–surface flux patches show signs of drift²⁹, whereas the VGP cluster patches suggest little movement over the past 10 Myr.

By definition, polarity reversal must involve weakening of the axial dipole field through zero. Although it is not yet understood

how the remaining dynamo-generated field (the non-dipole and equatorial dipole contributions) is affected by such a process, I now explore the structure of today's field stripped of its axial dipole. Figure 3 shows what would be the variation in the vertical component of the geomagnetic field at Earth's surface if the axial dipole (the g_1^0 term) were to completely vanish leaving the rest of the field unaltered. I claim that the structure of this g_1^0 -stripped field may hold important clues to understanding the source of the palaeomagnetic transition data.

Four principal features of the vertical field can be seen in Fig. 3. In the Southern Hemisphere, for example, there exists an intense downwardly directed field below the South Atlantic off the coast of Argentina, and an intense upwardly directed field over western Australia. These features show a strong correlation with the locations of the two palaeomagnetic transitional VGP cluster patches (Fig. 1a). Although this correspondence is striking, its explanation is not immediately obvious in that the palaeomagnetic data strongly suggest the cluster patches to be associated with dipole-dominated transitional field states. The vertical field features, on the other hand, are largely responsible for the more complex non-dipole part of today's field.

Not unlike the total field at the core–mantle boundary²⁷, the downward-continued g_1^0 -stripped field indicates that roughly beneath each vertical field feature in the Southern Hemisphere, and therefore beneath each VGP cluster patch, is a centre of strongly dipping flux emanating from the core, downwardly directed below the South America/South Atlantic cluster patch and upwardly directed below the western Australia cluster patch. Given this correspondence, one is tempted to simulate the inclined dipolar field configuration consistent with, say, the South America/South Atlantic VGP cluster patch by considering only the downwardly directed flux concentrated directly below it. I speculate that the strong correlation between the localities of Southern Hemisphere features of today's field, stripped of its axial dipole, and those of the VGP cluster data, from palaeomagnetic transition studies, may indicate that field sources which during times of stable polarity are largely responsible for the more complex non-dipole field may individually dominate during field reversal to produce long-lived inclined dipolar states.

If this is so, how might the western Australia VGP cluster patch form? This cluster patch may be less easily explained by today's field as this region of the core–mantle boundary is associated at present with concentrations of upwardly directed flux²⁷. For north VGPs to reside within the western Australia cluster patch, this bundle of near-radial flux would need to reverse (so as to become downwardly directed) as well as to dominate the reversing field at the Earth's surface. Hence, for the concentrations of near-radial flux associated with the Southern Hemisphere vertical field features seen in Fig. 3 to be independently responsible for the two observed VGP cluster patches seen in Fig. 1a, they must exchange sign and strength such that the feature with downwardly directed flux always dominates.

How such a dynamo process may take place is unclear; however, aspects of secular variation seem to be compatible with its occurrence. For example, localized features in the vertical component of the non-dipole field have been observed to grow or decay with time independently while remaining virtually fixed³¹. Also, palaeosecular variation recorded in lavas over the past 5 Myr shows a longitudinal bias to the distribution of VGPs⁸ roughly consistent with the two centres of near-radial flux discussed above.

Stationary field sources

The existence of transitional VGP cluster patches from lavas extruded over the late Cenozoic strongly suggests that the lower mantle exerts a significant influence on the core dynamo. Moreover, the relatively small size of each patch (Fig. 1a) suggests that variations about the core–mantle interface have

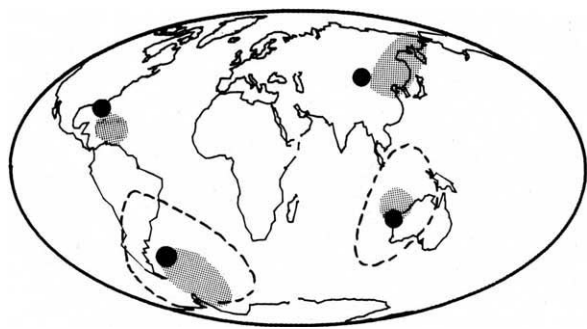


FIG. 4 Regions of fastest P-wave propagation in the lower mantle (shaded) (adapted from refs 5 and 32), localities of near-radial flux centres associated with today's surface field stripped of its axial dipole (●), and the transitional field VGP cluster patches derived from lavas. Although seismic velocity models determined from normal-mode data must have antipodal symmetry, the close pairing of velocity maxima with geomagnetic features provides credibility to the interpretation. That these paired observables are found well within the Southern Hemisphere VGP cluster patches suggests strongly that the sources of particular recurring inclined dipolar states during field reversal are tied to localized, long-standing dynamical features of the core–mantle system.

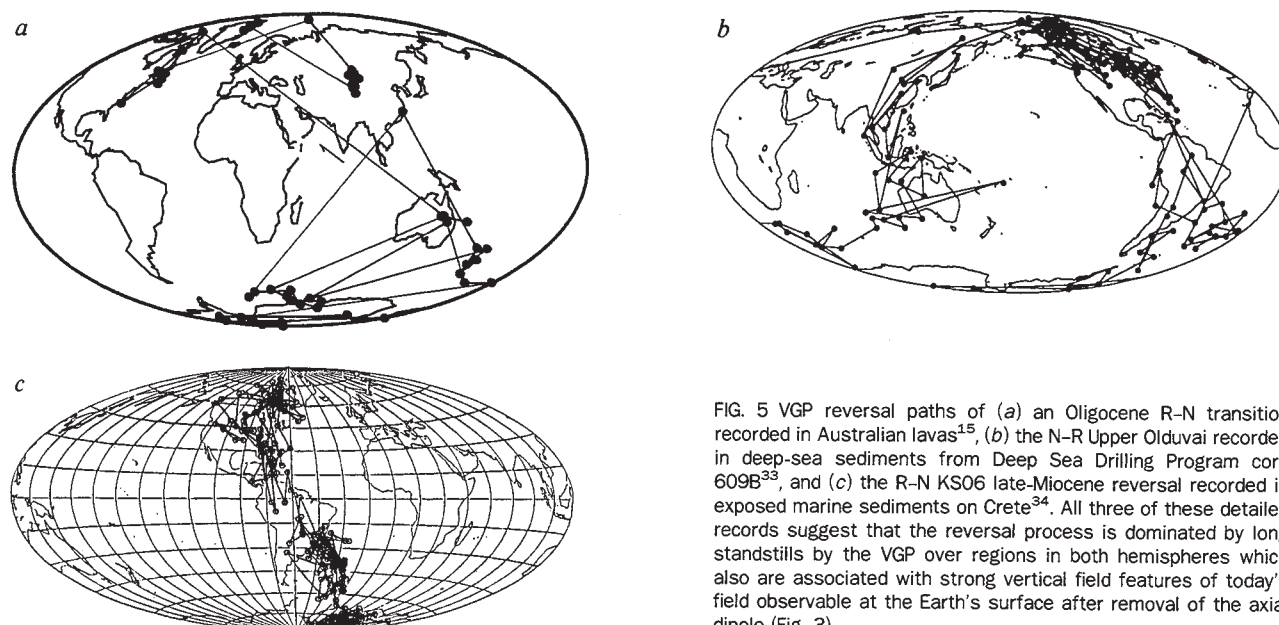


FIG. 5 VGP reversal paths of (a) an Oligocene R-N transition recorded in Australian lavas¹⁵, (b) the N-R Upper Olduvai recorded in deep-sea sediments from Deep Sea Drilling Program core 609B³³, and (c) the R-N KS06 late-Miocene reversal recorded in exposed marine sediments on Crete³⁴. All three of these detailed records suggest that the reversal process is dominated by long standstills by the VGP over regions in both hemispheres which also are associated with strong vertical field features of today's field observable at the Earth's surface after removal of the axial dipole (Fig. 3).

remained virtually invariant with respect to the Earth's surface for the past 10 Myr.

Laj *et al.*⁵ claim a rather remarkable correlation between the apparently preferred VGP banding observed in sediments and the pattern of anomalously fast seismic P-wave speed in the lower mantle³² deduced from normal-mode data. Although seismic velocity models derived from normal-mode data must have antipodal symmetry, allowing the possibility of artefactual agreement, this finding suggests that reversing field geometries are related to regions of cooler temperature in the Earth's deep interior.

Close inspection of these normal-mode results shows that each of the two bands of anomalously fast P-wave speed contains two regional maxima, one in the Southern Hemisphere and one in the Northern Hemisphere³². These four maxima are plotted in Fig. 4 along with the four localities of near-radial flux centres associated with today's surface field stripped of its axial dipole (Fig. 3). There is a clear one-to-one pairing of these features, and the two Southern Hemisphere pairs, in particular, lie well within the transitional VGP cluster patches (Fig. 1a). These striking associations suggest to me that what appear to be particular recurring inclined dipolar states during field reversal arise from localized, long-standing thermodynamic features of the core-mantle system.

Possible Northern Hemisphere correlations

Two vertical features of today's axial-dipole-stripped field are observed in the Northern Hemisphere, features of nearly equal significance to those found in the Southern Hemisphere. But in the north both features, one over the southeastern United States and the other over central Asia, involve intense downwardly directed field (Fig. 3).

That these northern field features and corresponding seismic P-wave speed anomalies (Fig. 4) may be associated with two additional inclined dipolar states during field reversal is suggested by the complex record of a R-N reversal about 34 Myr in age obtained from Liverpool volcano, Australia¹⁵, for which the VGP path is shown in Fig. 5a. Following what appears to be back-and-forth switching of the VGP between two localities in the Southern Hemisphere, each close to one of the VGP cluster patches defined by younger lavas (Fig. 1a), an unsuccessful attempt by the field to achieve normal polarity is recorded. Following this attempt the VGPs first cluster over eastern North America and then cluster over central Asia, both clusters occurring close to the Northern Hemisphere vertical field features

shown in Fig. 3. Also reported from Australasian lavas is a similar clustering of transitional VGPs over the eastern United States, the primary feature of a Miocene N-R reversal¹⁵. These older lava records suggest that each vertical feature seen in today's axial-dipole-stripped field (Figs 3 and 4), regardless of the hemisphere in which it is found, may in fact be associated with a distinct transitional inclined dipolar state. If so, then Northern Hemisphere VGP cluster patches may gain further definition as more lava records become available.

Lavas versus sediments

Reversal data obtained from lavas sometimes seem to be in conflict with those derived from sediments, but confirmation of my contention that particular long-lived inclined dipolar states dominate the reversal process must involve evidence from both types of recorders. Although a thorough analysis of sediment-derived data is beyond the scope of this article, I note here that some of the most detailed sediment records lend support to this claim.

Good examples are the reported VGP paths of the N-R Upper Olduvai transition obtained from deep-sea sediment core 609B³³ (Fig. 5b), and the KS06 R-N late-Miocene reversal record obtained from exposed marine sediments on Crete³⁴ (Fig. 5c). I choose these two reversal records because each is the most detailed dataset of a reported sequence of reversals from their respective sites and each contains more than one VGP cluster. As can be seen, both VGP paths indicate largely stop-and-go directional behaviour. The complex record from the 609B sediment core shows first a long standstill by the VGP over the eastern United States, and then to a lesser extent, quasi-stationarity within each of the two Southern Hemisphere VGP cluster patch localities (Fig. 1a). Also recorded is a rebound to the South Atlantic patch. The KS06 record indicates analogous behaviour, in that a long residence time by the VGP within the South American/South Atlantic cluster patch precedes an apparent standstill over the eastern United States.

These two well-known high-resolution sediment reversal records appear not to be in conflict with my interpretation of the lava-derived data. I suggest that analysis of the combined lava-sediment dataset may ultimately confirm the existence of long-lived, transitional dipolar field states and their association with structures observed in the present day field. If so, we may gain another useful handle to understanding the dynamical state of the core-mantle system. □

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A second-generation linkage map of the human genome

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A linkage map of the human genome has been constructed based on the segregation analysis of 814 newly characterized polymorphic loci containing short tracts of (C-A)_n repeats in a panel of DNAs from eight large families. Statistical linkage analysis placed 813 of the markers into 23 linkage groups corresponding to the 22 autosomes and the X chromosome; 605 show a heterozygosity above 0.7 and 553 could be ordered with odds ratios above 1,000:1. The distance spanned corresponds to ~90% of the estimated length of the human genome.

ABOUT 3,000 human polymorphic markers have been isolated¹, but 90% of them have a heterozygosity of less than 50% and they are unevenly spaced throughout the genome. Although maps based on these markers²⁻⁴ have contributed greatly to the primary mapping of a number of diseases, they are still insufficient for many applications such as mapping rare monofactorial diseases, refining linkage intervals to distances suited for gene identification, and mapping of loci contributing to complex traits^{5,6}. Therefore there is a need for more informative markers and a higher density linkage map.

Highly informative markers have been difficult to obtain. Although efficient strategies for the isolation of hypervariable minisatellite markers are available^{7,8}, these loci tend to cluster in the tips of chromosome arms. Recently however, the source of polymorphic markers has increased with the observation that short interspersed tandem repeats or microsatellites are frequently highly polymorphic, and can be analysed using polymerase chain reaction (PCR) techniques^{9,10}. Microsatellites appear widely distributed and can be readily isolated from genomic libraries.

The development of microsatellite markers on a large scale and the application of a multiplex genotyping procedure¹¹⁻¹³, allowed us to construct a genome map consisting mostly of markers with an elevated heterozygosity (>0.7) and covering a linkage distance spanning 90% of the genome as estimated from other maps⁴.

Development of microsatellite markers

DNA libraries were made from *AluI* DNA digests of 46,XX human DNA sized between 300 and 500 base pairs (bp) and

cloned in M13. With the exception of A_nT_n multimers, (C-A)_n-(G-T)_n repeats are the most frequent simple sequence repeats found in the human genome¹⁴. Recombinant plaques were therefore screened with a poly(dC-dA)-poly(dG-dT) probe. DNA templates from (C-A)_n-positive clones, ~1% of the M13 plaques were then sequenced and amplification primers on either side of the poly A-C tract were chosen using a computer program. From a total of 12,014 sequenced inserts, 2,995 were selected for amplification assays; 9,028 clones were discarded for assorted reasons (Table 1) including clones for which the C-A repeat was too short (<12 units long) or absent, or beyond the reliable part of the determined sequence. In some other sequences no oligonucleotide pairs fulfilling our requirements for primer selection could be found.

Primer pairs were synthesized for the 2,995 selected microsatellite markers and assayed by PCR amplification of four unrelated 46,XX individuals from the CEPH families. This assay was also used as a first rough estimate for the polymorphism of the tested microsatellites and to favour selection of highly informative markers. To aid large scale genotyping, a single set of PCR conditions was chosen¹³. Among 2,995 markers tested on the four individuals, 84% (2,506) were successfully amplified, 93% (2,327) of the amplified markers were polymorphic, 72% (1,809) showed three or more alleles and in 36% the highest allele frequency was below 0.5. About 4% of primer pairs promoted amplification of more than one allelic system. Only those showing unambiguously resolved systems were used further.

Markers with three or more alleles were first assigned to their chromosome using DNAs from a panel of cell lines of 18 human rodent hybrids each containing a subset of human chromosomes.

TABLE 1 Overall recovery of (C-A)_n containing M13 templates

Type of sequence	Number of sequences	Per cent of total
Sequences used for PCR	2,995	25
Primers not found	624	5
8 to 12 (A-C) repeats	1,398	12
Microsatellite absent of <8 (A-C) repeats	956	8
Unreliable sequences	2,750	23
Misplaced microsatellites	2,910	24
Duplicates	381	3
Total	12,014	100

Sequencing was done using the dideoxynucleotide chain termination method with fluorescent primers¹⁹ on Applied Biosystems 373A DNA sequencers. Sequencing reactions were done using the *Taq* polymerase linear amplification procedure (cyclic sequencing)²⁰ according to a protocol established by ABI. A single sequencing run was done for each template, and unreliable sequences were discarded. Oligonucleotide primers flanking the microsatellite tandem repeats were chosen with a computer program derived from the program OLIGO²¹ and using additional criteria like base composition, avoidance of homologies with repeated sequences (Alu, Kpn and so on). Their *T_m* ranged from 55 °C to 70 °C. Special care was taken to avoid self priming, particularly at the 3' ends. The primers were purchased from a company (Genset, Paris) and were used without purification for PCR reactions.

We found that 1,547 markers (86% of 1,806 tested) could be assigned to a chromosome (Table 2). The overall microsatellite density per chromosome fluctuates between 0.57 and 1.53 times the mean value of 0.49 marker per megabase (Mb). Some chromosomes such as 21, 9 and 19 contain significantly less (C-A)_n than average whereas others such as 17 or 18 contain much more.

A better estimation of observed heterozygosity values of these 814 markers was based on scoring 28 unrelated CEPH individuals. Heterozygosities range from 0.32 to 0.96. 790 markers (97%) show a heterozygosity above 0.5 and 605 (74.4%) above 0.7 (Fig. 1).

Genotyping and data verification

The potentially most informative markers were genotyped on a subset of the CEPH panel composed of the eight largest families. This genotyping was extended to less informative markers for chromosomes with lower microsatellite densities. The eight families contained a total of 91 children, 16 parents and 26 grandparents. Genotypes were characterized following a multiplexing procedure that we have developed recently¹⁵. Results were obtained as images on films after photographic exposures of hybridized membranes¹³. Allelic systems with unclear or poorly resolved amplification patterns were discarded. The genotypes were interpreted in two independent readings of the images, and entered twice into a computerized database. The two entries were compared, and differences were either resolved by a third interpretation, or the data were judged to be ambiguous and removed. Further verifications of the data were made in families that were identified as containing possible genotype errors based on the pattern of pairwise recombination events between linked markers.

Several segregation anomalies were encountered. In some families absence of amplification of one allele could be deduced from the segregation analysis. Such an allele was considered as cryptic and these genotypes were discarded. Mutations have been observed for 160 markers. In most cases they affected a single allele from one individual (120 markers) or one allele in two generally unrelated individuals (27 markers). Thirteen markers showed between three and seven mutation events. But because these latter events were more frequently found in a single family, a number of those may be ascribed to germ-line mosaicism and not to independent events. Altogether these

different events correspond to a mutation rate close to 0.1%. Genotypes of individuals with obvious mutations were eliminated. Note that due to the rather important mutation rate of microsatellites one allele may sometimes be converted into another allele existing in the family, resulting in an erroneous interpretation of the origin of an allele.

Linkage and genetic maps

Genotypes in the eight families were analysed with the LINKAGE programs¹⁵. All 814 markers showed significant linkage (lod score >3) to at least three other markers in the CEPH database (version 5). Generally, the linkage results and the physical assignments based on somatic cell hybrids were in agreement. But, in a few instances (3%) the physical assignments were found to be erroneous. All markers that were not physically assigned (~15%) could be assigned through linkage, except for a probably pseudoautosomal marker. Pairwise lod scores were calculated between markers assigned to the same chromosome, and the recombination estimates were used to choose a subset of 5–18 loci with spacing of ~10 to 20 centimorgans (cM) as a framework map for each chromosome. In some instances some larger intervals had to be taken. The order of the framework markers was confirmed with odds >1,000:1 against possible alternatives, and the framework maps were used as a starting point to order other markers.

As expected, an overall larger genetic length was observed in female meioses (data not shown). The complete maps for all chromosomes are represented in Fig. 2 with the sex-average recombination distances between adjacent markers. Rough localizations for selected reference markers from the CEPH database^{4,16} are shown to allow comparison to other genetic maps. The markers on each chromosome from a single linkage group with the exception of chromosome 12, for which the most

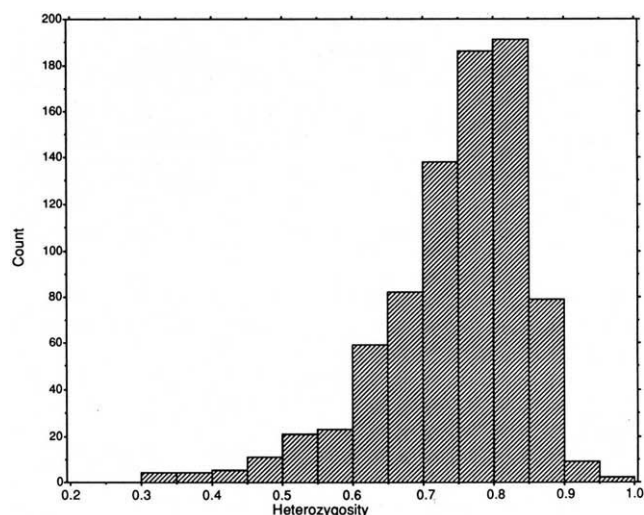
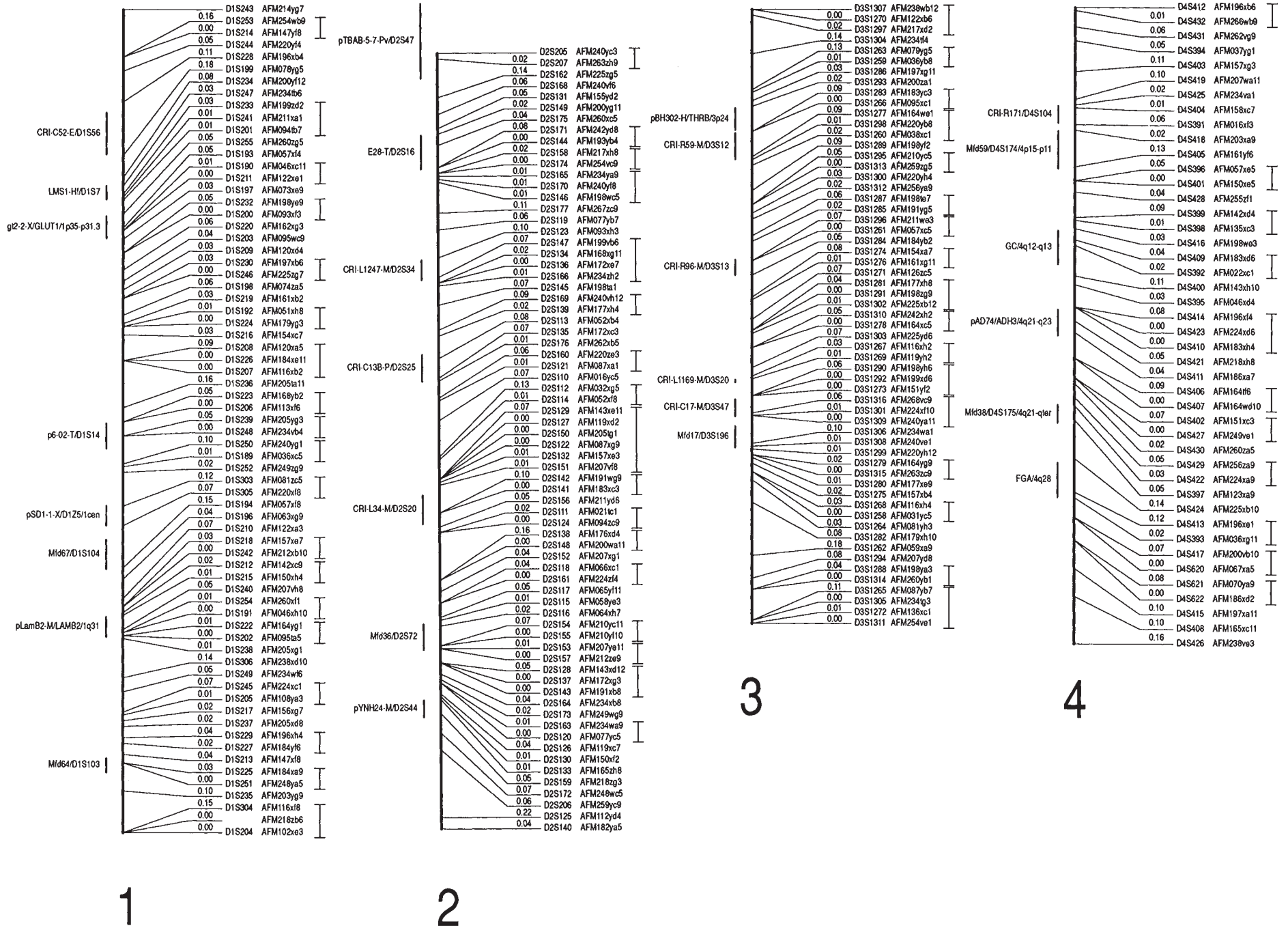
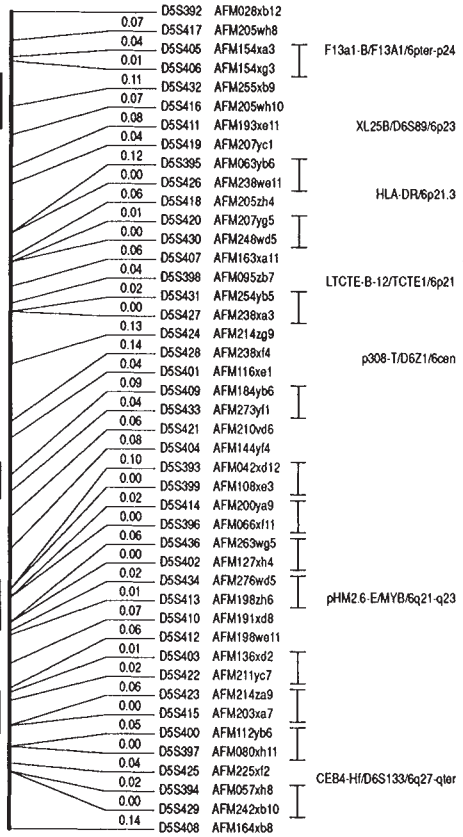


FIG. 1 Heterozygosity of 814 (C-A)_n microsatellites mapped in this study. Heterozygosity was estimated from a total of 28 unrelated individuals of the eight largest CEPH families (CEPH pedigree numbers 102, 884, 1331, 1332, 1347, 1362, 1413 and 1416). DNA samples were amplified by PCR independently for each marker and were amplified as described¹³. The reactions were performed using a 'hot-start' procedure: *Taq* polymerase was added only after a first denaturation step of 5 min at 96 °C. Eight to sixteen different PCR products amplified on DNA samples from the same individual, were coprecipitated and comigrated in a single gel lane. About 80 coprecipitates (four families) were processed on a single gel. Coprecipitated PCR products of the M13 templates cognate with the typed markers were used as sizing standards along with ladders of poly(A). Products separated on denaturing acrylamide gels were transferred onto Hybond N⁺ nylon membranes. Membranes were successively probed with nonradioactively labelled PCR primers, as reported¹³. Primer sequences and allele frequencies of these markers have been deposited in the GDB.

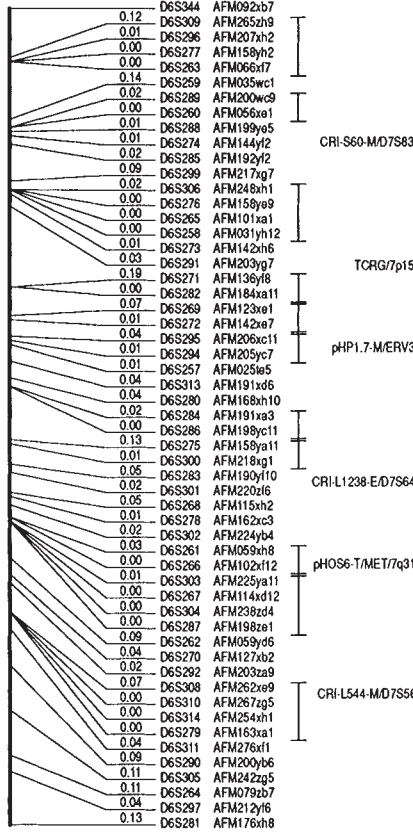
Sex-averaged linkage maps of 814 microsatellite markers



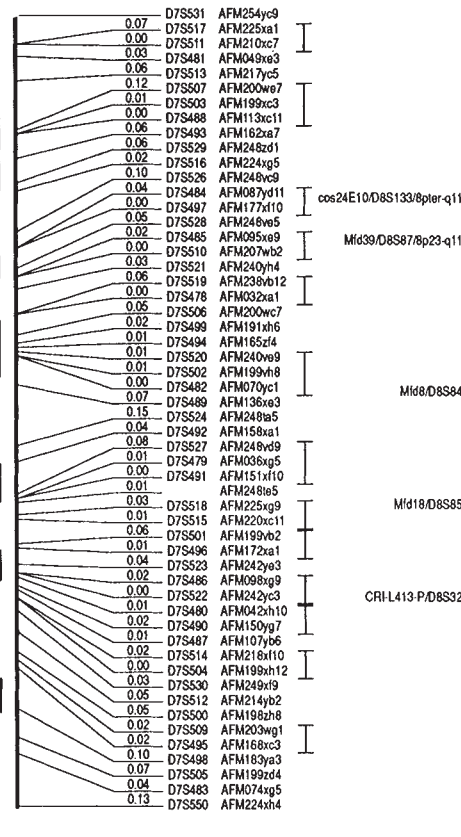
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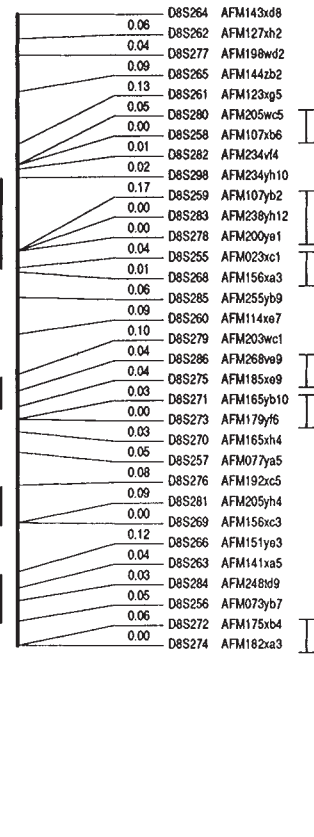
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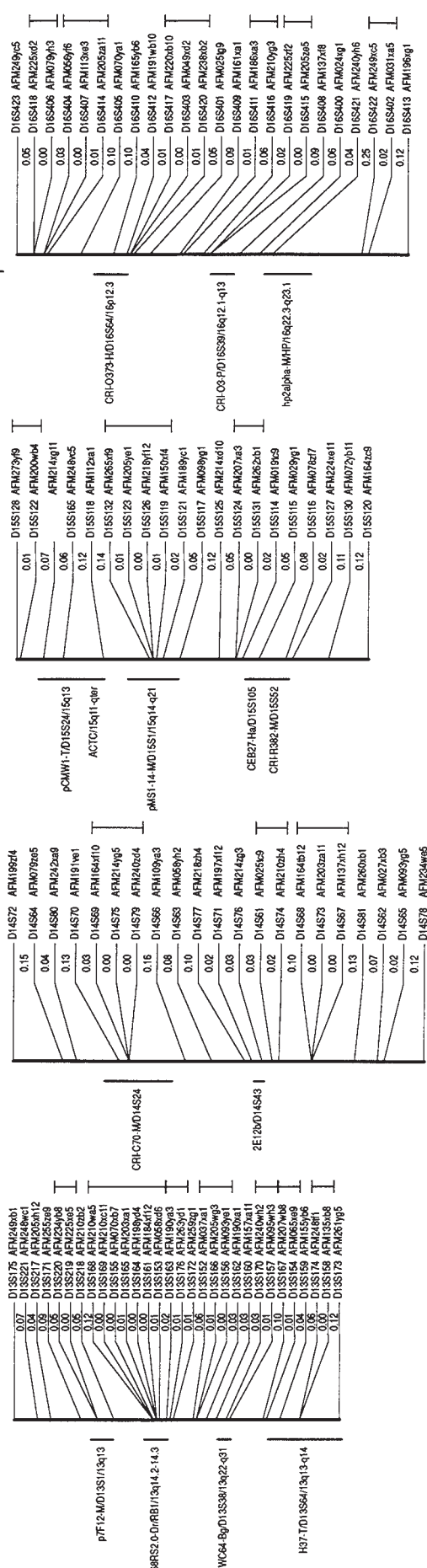
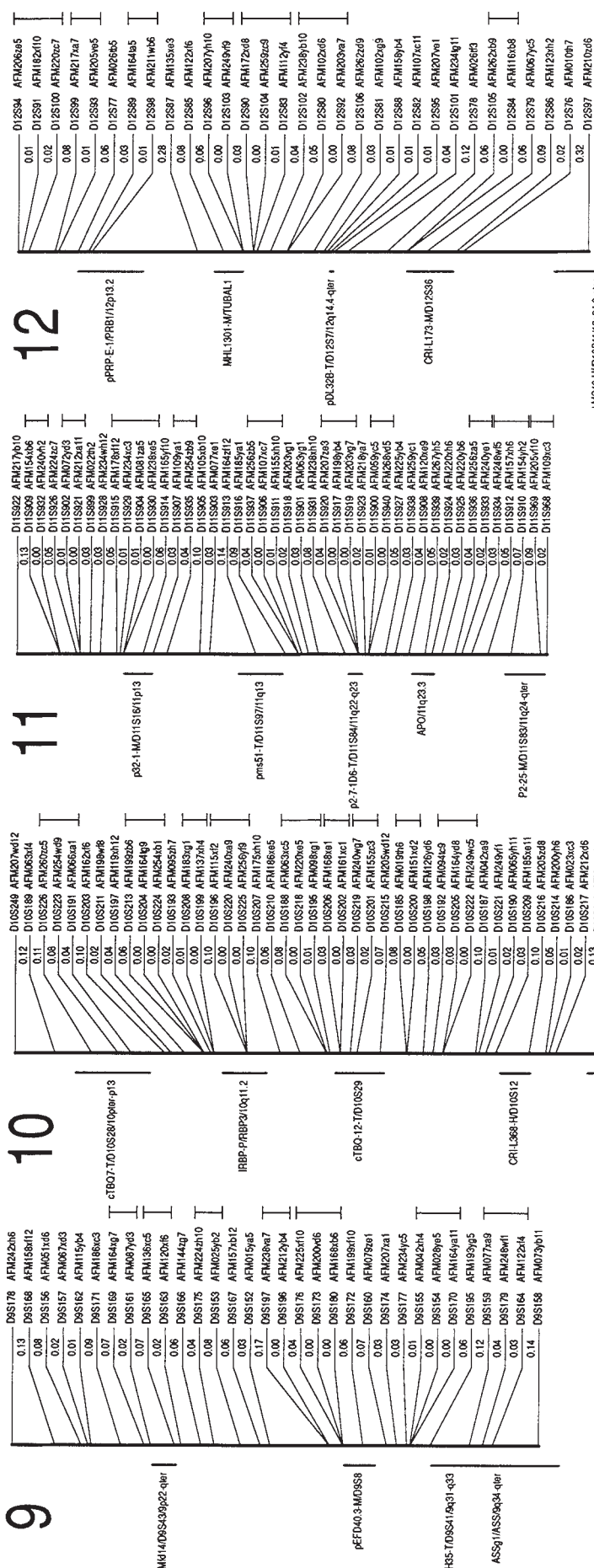


FIG. 2 Chromosome maps showing the best-supported order of the markers, and sex-average recombination fractions between adjacent markers. Maps are shown to scale except for chromosomes 21 and 22. Groups of markers for which the order cannot be resolved with odds >1,000:1 are indicated by solid lines beside the names of the loci (on the right of each chromosome map). Rough locations are shown for selected reference markers from the CEPH database (version 5) on the left of the chromosome maps. The bars indicate regions of 1,000:1 odds for positions of the reference markers based on location score analysis with respect to our maps. Physical assignments and mapping data for reference markers from the CEPH database were taken from the chromosome reports in Human Gene Mapping 11¹⁶ and from the NIH/CEPH comprehensive linkage map listed in ref. 4. The order of markers was determined as described²³. Error detection was done before map construction based on both pairwise and multilocus recombinant analysis²⁴.

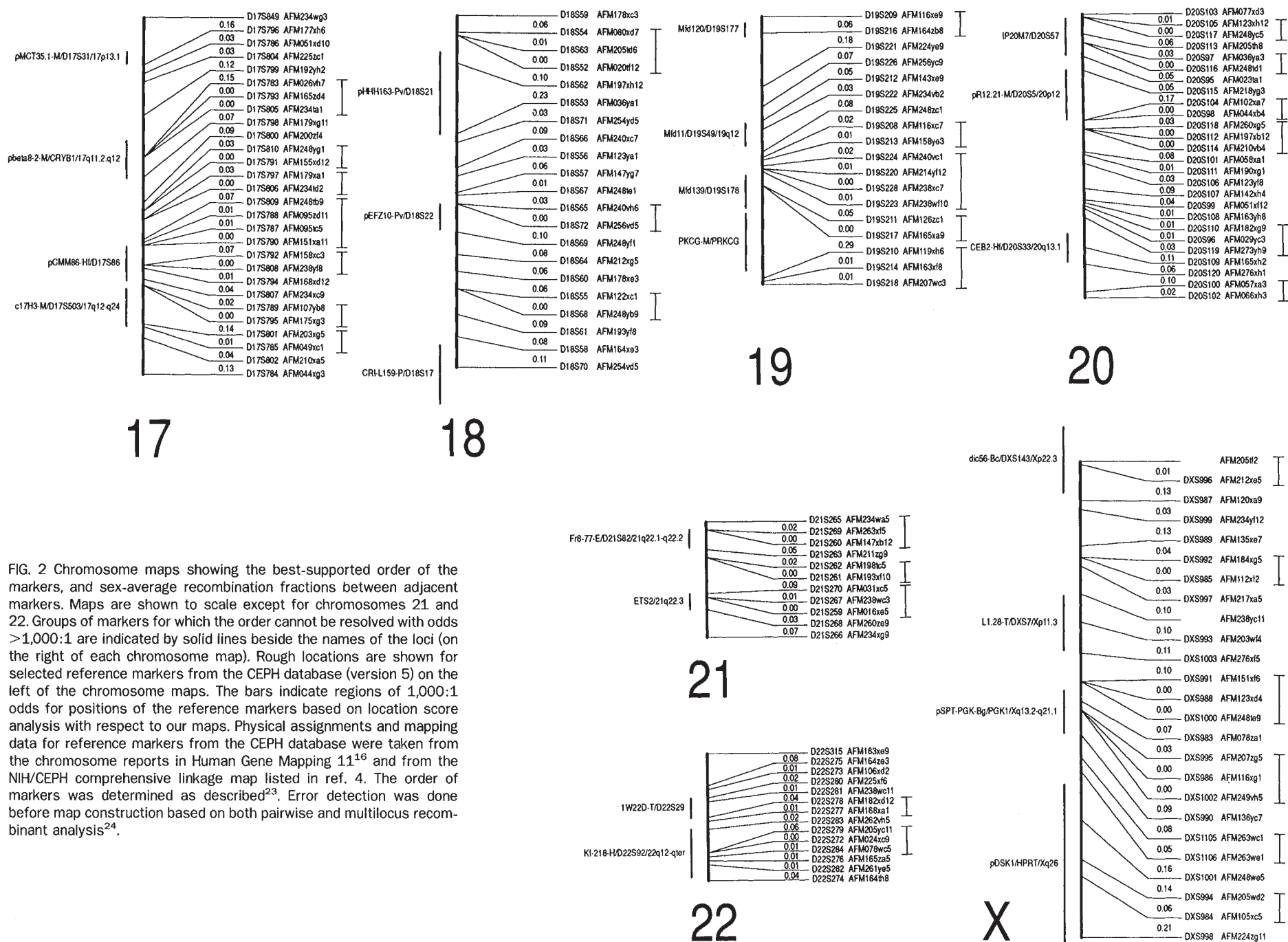


TABLE 2 Summary of quantitative characteristics of the chromosome markers and maps and comparisons to reference data

Chromosome	Physical length (Mb)	Number of markers assigned	Markers per Mb assigned	Estimated genetic length* (cM)	NIH/CEPH genetic length† (cM)	Length covered (cM)	NIH/CEPH extra length pter (qcen)‡ (cM)	NIH/CEPH extra length qter (cM)	Number of markers mapped	Number of markers positioned with odds >1,000:1	Mean interval (cM)§
1	263	122	0.46	305	390	295	19	22	69	48	4.28
2	255	126	0.49	271	354	277	2	0	70	47	3.96
3	214	105	0.49	237	334	221	10	10	59	35	3.75
4	203	85	0.42	244	275	229	3	10	44	32	5.20
5	194	97	0.50	234	297	201	3	4	44	31	4.57
6	183	104	0.57	207	249	201	10	10	55	36	3.65
7	171	80	0.47	178	267	195	7	10	54	38	3.61
8	155	86	0.55	172	237	155	0	25	32	25	4.84
9	145	45	0.31	146	170	160	2	6	32	22	5.00
10	144	83	0.58	181	250	178	5	3	42	28	4.24
11	144	72	0.50	150	245	161	-4	20	44	29	3.66
12	143	71	0.50	160	201	172	-5	0	31	21	5.55
13q	98	46	0.47	130	219	99	25	45	30	17	3.30
14q	93	54	0.58	122	171	125	4	38	21	16	5.95
15q	89	46	0.52	154	178	107	7	2	20	15	5.35
16	98	42	0.43	157	162	119	10	8	24	16	4.96
17	92	69	0.75	208	168	128	2	12	28	18	4.57
18	85	53	0.62	143	109	126	-30	0	21	17	6.00
19	67	23	0.34	148	114	95	15	10	18	10	5.28
20	72	40	0.56	122	147	101	0	37	26	18	3.88
21q	39	11	0.28	114	67	29	15	10	11	6	2.64
22q	43	20	0.47	81	98	32	37	25	14	11	2.29
X	164	67	0.41	220	208	170	≈0	10	25	17	6.80
Total	3,154	1,547	0.49	4,084	4,910	3,576	176	317	814	553	4.39

The extra length of the NIH/CEPH maps has been evaluated using, as a reference point for each telomeric region, a marker from the CEPH database very closely linked to the most distal marker from our maps. To aid linkage analysis, markers were first physically assigned to chromosomes when possible. DNA from cell lines from a panel of 18 human rodent hybrids (NIGMS Human Genetic Mutant Cell Repository panel 1, Coriell Institute, Camden), each containing a subset of human chromosomes, were tested by PCR for all tri- or multiallelic markers and scored for the presence or absence of an amplification product. PCR amplifications were done as described¹³. PCR products were visualized on agarose gels.

* Values from ref. 22.

† Values from ref. 4.

‡ For the acrocentric chromosomes (13, 14, 15, 21, 22), this extra length applies to qcen and not pter.

§ Mean interval is the ratio of length covered per number of markers mapped.

|| Position of the distal marker framing the largest gap of chromosome 12 (see Fig. 2) is not supported by odds of 1,000:1 and has not been included in the distance covered by this linkage group.

distal marker (AFM210zd6) is not significantly linked to the other markers. But AFM210zd6 has lod scores >12 with two other markers from the CEPH database that had previously been mapped to chromosome 12q. A subset of 553 of the 814 markers formed an anchor map in which the order of all markers were resolved with odds >1,000:1, demonstrating the power to obtain precise linkage maps from highly informative markers in the eight families we selected for genotype characterization.

Table 2 summarizes several other features of the linkage maps. The average distance between adjacent markers is ~5%, and the total distance spanned by the markers was estimated to be 36 Morgans (Kosambi's mapping function). A total of seven internal intervals larger than 20 cM between adjacent loci can be seen on Fig. 2. In addition several subtelomeric regions appear to have a low density of markers (for example 1q, 2q, 12q, 20q). Conversely, clusters of microsatellites are observed in several regions. In particular, 17 of 30 markers on chromosome 13 are localized within a 24 cM interval. The maps for several of the smaller chromosomes, such as 21 and 22, cover less than 40% of their estimated length, which also suggests clustering of microsatellite markers on these chromosomes.

Discussion

We isolated and mapped a large number of (C-A)_n microsatellites to construct a linkage map of the human genome with an average resolution of 5 cM. The quality of such a map relies on the informativeness of the markers and their distribution.

The mean level of heterozygosity of our markers is close to

0.75 indicating that the selection procedure for highly polymorphic markers has been successful. This procedure was based on (1) selection of markers containing a minimum of 12 C-A doublets in the sequenced allele, (2) test of polymorphism on a sample of four individuals (eight alleles), (3) choice of markers for which the highest allele frequency was <0.5 among these eight alleles. Use of these last criteria has been evaluated recently¹⁷.

Although the markers obtained are widely dispersed, two sets of arguments indicate that their distribution is not random. First, the distribution observed by chromosome assignment of 1,547 markers selected only on the basis of potential informativeness has an almost threefold variation between different chromosomes adjusted to their physical size. Because the source library of the markers was constructed using size fractionated *AluI* fragments, it could be argued that it is actually the sequence of *AluI* restriction sites (A-G-C-T) which is non-uniformly distributed among chromosomes. But in both cases this indicates that some sequence motifs do not have the same frequency on all chromosomes. Tentative correlations of this non-uniform distribution with the ratios of G to R bands of each chromosome were not conclusive. Otherwise a bias in distribution could result from the various selection steps such as exclusion of less informative or poorly amplified sequences or sequences for which no primers could be selected. It should be noted that the first set of markers (~760) was selected on the basis of high informativeness. Some less informative markers mapping to poorly covered chromosomes (for example 13, 21, 22 and so on) were included afterwards. Several other markers were added to gaps

larger than 20 cM or to extend the lengths covered for some chromosomes.

Second, the non-random distribution within a single chromosome is sometimes even more dramatic, as best exemplified by chromosomes 13 and 22. Because the cytogenetic location of our markers is unknown, correlations between physical and linkage distances is speculative. But an apparent depletion of microsatellites in terminal parts of chromosomes was found for chromosome 20 (ref. 12) and might also be related to the map inflations observed in terminal regions.

The NIH/CEPH chromosome maps include 1,416 markers and most frequently extend slightly beyond our maps. Because these map extensions amount approximately to 5 Morgans (Table 2), our maps cover about 90% of the NIH/CEPH maps. A discrepancy of 8 Morgans still remains between the two sets of data. This discrepancy varies from chromosome to chromosome as can be seen from Table 2 but might be in part attributable to the heterogeneous set of data used to construct the

NIH/CEPH map and to the fact that the present maps are based on a subset of eight families from the CEPH panel.

Despite several gaps above 20 cM, the present maps provide a general tool which should allow primary linkage mapping of most single gene disorders. This should be possible even when only a limited number of affected families is available because of the high informativeness of the markers. This collection of markers should also serve as a resource to help fill the remaining gaps in the present effort to construct index marker maps with heterozygosities above 0.7 and no gaps greater than 15 cM. Note that many of the gaps correspond to subtelomeric regions in which most of the known variable number of tandem repeats loci reside. Thus the two alternative sources of micro- and minisatellites appear to be complementary, and the existing markers from these sources together may constitute at present an almost continuous set of highly informative markers covering the human genome. A mouse genetic map of similar resolution has been reported recently¹⁸. □

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Crystal structure of staphylococcal enterotoxin B, a superantigen

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The three-dimensional structure of staphylococcal enterotoxin B, which is both a toxin and a superantigen, has been determined to a resolution of 2.5 Å. The unusual main-chain fold containing two domains may represent a general motif adopted by all staphylococcal enterotoxins. The T-cell receptor binding site encompasses a shallow cavity formed by both domains. The MHCII molecule binds to an adjacent site. Another cavity with possible biological activity was also identified.

THE staphylococcal enterotoxins comprise a family of five serologically distinct toxins (labelled A-E), all of which are secreted by various strains of *Staphylococcus aureus*, and share significant sequence homology¹⁻³. These toxins are primary causes of food poisoning, with all members inducing the same biological effects: emesis, diarrhoea and mitogenesis¹⁻⁵. More importantly, they can induce profound immune system responses by stimulating T cells with particular V β elements⁶⁻¹⁰. The fraction of responding T cells can be orders of magnitude greater than that evoked by conventional antigens. Accordingly, staphylococcal enterotoxins have been termed 'superantigens'¹¹.

Superantigens have similarities to conventional antigens. For example, both types of exogenous antigens must be presented to a T-cell antigen receptor (TCR) by a class II molecule of the major histocompatibility complex (MHCII)^{12,13}, and both types

form ternary complexes (antigen-MHCII-TCR) that trigger cytokine production and T-cell proliferation. But superantigens and antigens differ in some main respects. The main differences lie in how they interact with MHCII and TCR molecules. Ordinary antigens are preprocessed into short peptide fragments, 13 to 17 residues in length¹⁴, that bind to a cleft on the surface of the MHCII molecule for presentation to the TCR^{15,16}. In contrast, with superantigens the intact protein molecule binds to MHCII¹². There is also evidence that superantigens bind to a different place on the MHCII molecule than do processed peptides¹⁷⁻¹⁹.

The difference in the mode of binding gives rise to different T-cell interactions. When a peptide-MHCII complex combines with a TCR, the antigenic fragment interacts with variable components (V) and others on both TCR α and β chains²⁰. The

very small number of such TCR combining sites that are complementary to a given peptide fragment leads to a low, controlled response. In contrast, for superantigens a ternary complex has been suggested that places the superantigen in contact with amino-acid residues on surfaces of the TCR V β region and the MHCII molecule that are external to the peptide-binding cavity²¹. Because the superantigen-MHCII complex interacts primarily with V β components on the TCR, it can stimulate all TCRs bearing only the appropriate V β , regardless of the other components. As there are a limited number of V β elements (<30 for any given human), this represents a large proportion of the total T-cell population, accounting for the massive T-cell proliferation and enhanced cytokine release.

Superantigenicity may present therapeutic possibilities as well. For example, injecting mice with staphylococcal enterotoxin B (SEB) can reduce the severity of lupus nephritis, an autoimmune disorder, and treating animals with the toxin can protect them against experimental multiple sclerosis²². The three-dimensional crystal structure of SEB, perhaps the most widely studied member of the staphylococcal enterotoxins, is reported here. It is the first superantigen for which detailed structural information is available.

Structure determination

Details of the structure determination and refinement statistics are given in Tables 1 and 2. The electron density map was of high quality except for a seven-residue region in the disulphide loop. Omit maps computed with $2F_o - F_c$ as coefficients reveal density for the omitted residues (Fig. 1). All non-glycine residues are within the allowed regions in the Ramachandran plot²³ except for 10 residues, 7 of which are in the disulphide loop where the density is poorly defined. The spatial organization of the model explains the distribution in the amino-acid sequence of the residues that are critical for TCR interaction; they coalesce into one site. Also, all residues implicated in MHCII binding are located on the molecular surface.

General topology of the structure

The SEB molecule contains two domains. The first is composed of residues 1–120, although the 29 residues at the amino terminal are more closely associated spatially with the second domain which comprises residues 127–239. A schematic drawing of the folding pattern and a ribbon drawing²⁴ are shown in Fig. 2a and b, respectively. Domain 1 contains two β -sheets, one formed by $\beta 1$, $\beta 4$ and $\beta 5$ and another consisting of $\beta 2$, $\beta 3$, $\beta 4$ and

TABLE 1 Structure determination data

Data set	Resolution (Å)	Unique reflections (%)	R_{merge} (%) [*]	R_{iso} (%) [†]	Number of sites	R_c (%) [‡]	Figure of merit	Phasing power [§]
Native	2.50	8,970 (95.5)	6.5					
Pt-diaminodichloride	2.75	5,959 (87.7)	5.9	10.1	6	47.0	0.474	2.09
PIP	2.75	6,409 (94.3)	7.3	15.8	4	40.0	0.373	1.65
Bakers mercury	3.00	4,459 (84.6)	11.0	20.0	5	51.0	0.335	1.46
TAMM¶	3.00	4,663 (88.5)	8.0	14.1	5	54.0	0.255	1.28
Mercuric chloride	3.00	4,505 (85.5)	9.0	20.4	3	56.0	0.245	1.20
PIP and dimercuric malonic acid	3.00	4,891 (92.8)	8.0	17.4	6	54.0	0.354	1.88
Pt-diaminodichloride (anomalous)	3.50	2,101 (62.2)	8.5		5	36.0	0.396	1.71

Crystallization as described³¹. Single crystals of SEB were grown by vapour diffusion against PEG 4,000, pH 8.8. There are three crystal forms that differ slightly in their axial lengths, though they have a similar rectangular plate morphology (except that Form II crystals are rod-like on occasion). Unit Cell, Form I: $a=45.3$ Å, $b=70.6$ Å, $c=78.1$ Å; Form II: $a=45.5$ Å, $b=68.3$ Å, $c=79.4$ Å, Form III: $a=45.4$ Å, $b=71.2$ Å, $c=78.4$ Å; Space group $P2_12_12_1$, $Z=4$; M_r 28,400K; Matthews's coefficient³¹, 2.2 Å³ per Dalton. All data were collected with a Siemens area detector on a Rigaku RU200 rotating anode generator with a 0.2 × 2.0 mm focus. CuK α radiation was provided by a Frank's double focusing mirror assembly. All data were processed using the Xengen³³ package, and scaling of the data was done using a program modified from ref. 34. Native data sets were collected from all three forms. The disagreement in the structure factors of the three forms (R factors on intensities ranging from 26% to 38%) clearly indicated that the forms are quite distinct. Structure determination was done using diffraction data from Form II crystals. Data were collected for several derivatives. A problem with heavy-atom soaking was that the cell dimensions sometimes changed, suggesting a transformation of the crystal form, caused either by non-isomorphism, another crystal form or phase transformation. Therefore, derivative data were used only if the cell dimensions agreed with Form II native crystals within experimental errors. Seven isomorphous and two anomalous data sets were collected. Anomalous data were collected according to ref. 35, aligning the crystal with its a -axis along the omega-axis. Phase Calculation: Three heavy-atom sites were initially located from the Patterson map for a platinum derivative and the rest using a difference Fourier map. The heavy-atom sites for the other derivatives were determined using a combination of Patterson and cross-phased Fourier methods. The heavy-atom parameters were initially refined against centric data. The phases were then combined to give multiple isomorphous replacement with anomalous scattering (MIRAS) phases. Even though the data for most of the derivatives extended to 2.75 Å resolution, the initial MIRAS phases were only calculated to 3.5 Å resolution because of non-isomorphism of the derivative crystals or the drop in phasing power at higher resolution. The overall figure of merit was 0.74. The phases were improved by 16 cycles of solvent levelling^{36,37} using 40% solvent content; the phases were further extended to a resolution of 2.5 Å. In general, the secondary structural elements were obvious but the connecting segments were not clear. The heavy-atom parameters were further refined against the solvent-levellied protein phases to obtain better phases, that allowed location of extra heavy-atom sites for some derivatives and clearly indicated that two data sets, one isomorphous and one anomalous (both from the same heavy-atom reagent) were not consistent with the phases from the other derivatives; the phasing power of both dropped to a value <1.0, and accordingly were excluded from the phase calculations. A new MIRAS map was then computed and solvent flattened. All heavy-atom refinement, MIRAS, solvent flattening and map calculations were computed with the program package PHASES³⁸. The final solvent-levellied map with only seven derivative data sets included in the phase calculation was of higher quality than the first. The side chains were mostly well defined. The starting C α chain was traced using 'mini-maps' and the bones trace from the program FRODO^{39,40}. The most probable side chains were assigned to each of the C α atoms traced. The sequence alignment was done with the slider option in Program 'O'⁴¹ and visual inspection of the trace. Two models, slightly differing in sequence⁴² register, were tested by refining with X-PLOR⁴³, and the model that gave a significantly better crystallographic R -value chosen as the correct model. (Electron density map, see Fig. 2a; refinement; see Table 2.) Residues 99–105, missing in the disulphide loop, were built in stereochemically to get a continuous chain, though these residues could not be located even in the final $2F_o - F_c$ map. Combined FOM, 0.745 (3.5 Å data); after solvent flattening FOM, 0.896; extending to 3.0 Å resolution FOM, 0.820; extending to 2.5 Å resolution FOM, 0.660.

^{*} $R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i \langle I \rangle$ where I_i is the intensity measurements for a reflection and $\langle I \rangle$ is the mean intensity for this reflection.

[†] $R_{\text{iso}} = \sum_i |F_{\text{PH}}^2 - F_{\text{P}}^2| / \sum_i (F_{\text{PH}}^2 + F_{\text{P}}^2)$ where F_{PH} and F_{P} are the derivative and native structure factors, respectively.

[‡] $R_c = \sum_i |F_{\text{PH}} \pm F_{\text{P}}| - |F_{\text{H}}(\text{calc})| / \sum_i |F_{\text{PH}} \pm F_{\text{P}}|$ for centric reflections.

[§] Phasing power = $\langle F_{\text{H}} \rangle / E$; the r.m.s. heavy-atom structure factor amplitude divided by the residual lack of closure error.

|| di- μ -iodobis(ethylene diamine)diplatinum (II) nitrate.

¶ tetrakis (acetoxymethyl) methane.

FIG. 1 ($2F_o - F_c$) omit map showing a region rich in hydrophobic residues. Only those residues omitted in the map calculations are shown.

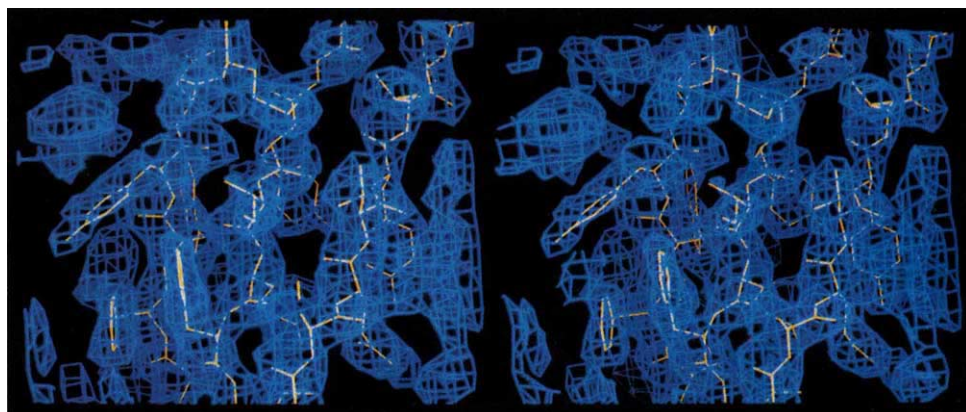


TABLE 2 Refinement statistics

<i>R</i> -value*	0.193
Data used	8 Å to 2.5 Å
R.m.s. deviations	
Bond lengths	0.020 Å
Bond angles	3.5°
<i>R</i> free value†	0.354

The low angle data were not included in the refinement to compensate for non inclusion of solvent molecules in the model. Unrestrained individual temperature factor refinement was used in the final cycle.

* Final value for reflections with $I > 2\sigma(I)$ after several cycles of X-PLOR refinement and manual intervention.

† See ref. 44.

$\beta 5$. It also contains three α -helices: $\alpha 1$, $\alpha 2$ and $\alpha 3$. Strands $\beta 4$ and $\beta 5$ curve around to form part of both sheets. The two sheets form a cylindrical barrel whose inner wall is lined with hydrophobic residues and whose ends are covered by $\alpha 3$ and a loop (54–60). On a projection normal to the axis of the cylinder the two β -sheets form a 'criss-cross' pattern. The disulphide loop, which is a part of this domain, extends into the solvent with residues 99–105 apparently disordered.

Domain 2 comprises two distinct parts. One part consists of two α -helices ($\alpha 4$ and $\alpha 5$) and two very short β -strands ($\beta 8$

and $\beta 11$). The lower half of $\alpha 4$ interacts with the upper half of $\alpha 5$, whereas the upper half of $\alpha 4$ interacts with $\alpha 2$. A twisted β -sheet formed by strands $\beta 6$, $\beta 7$, $\beta 9$, $\beta 10$ and $\beta 12$ completes the domain. Strand $\beta 12$ is parallel to $\beta 6$. The crossover from $\beta 6$ to $\beta 12$ is an infrequently observed left-handed type²⁵. The α -helical part lies between the twisted sheet and the β -cylinder, which is unusual in that helices are most frequently found on the exposed faces of β -sheets. But both $\alpha 4$ and $\alpha 5$ have one side exposed to the solvent, and are associated with grooves on opposite sides of the molecule.

Structural features of superantigenic regions

Three regions have been identified²⁶ (Fig. 2b) in the N-terminus part of SEB that affect MHCII binding and/or T-cell activation. In each of the regions the specific amino acids that are responsible were determined. Some of the identified residues affect both MHCII binding and T-cell activation, whereas others affect only the latter. As superantigen-MHCII binding is a prerequisite for T-cell activation, residues affecting MHCII binding will also influence T-cell activation, thus no T-cell binding information can be inferred from them. But they do provide information about MHCII-binding sites on the superantigen. On the other hand, those residues that influence T-cell activation but not MHCII binding are likely to be in the T-cell binding site on SEB. Interpretation of the three-dimensional structure in this

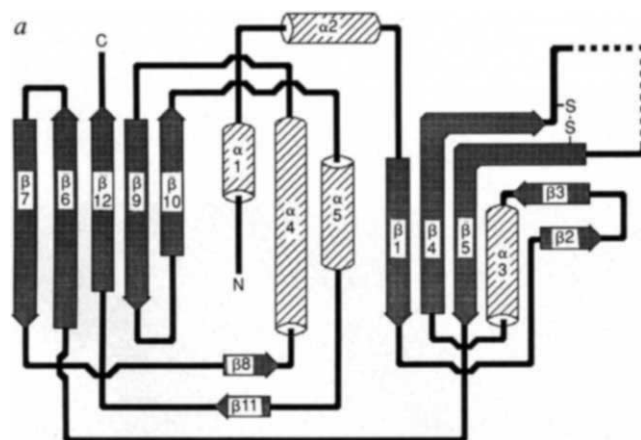
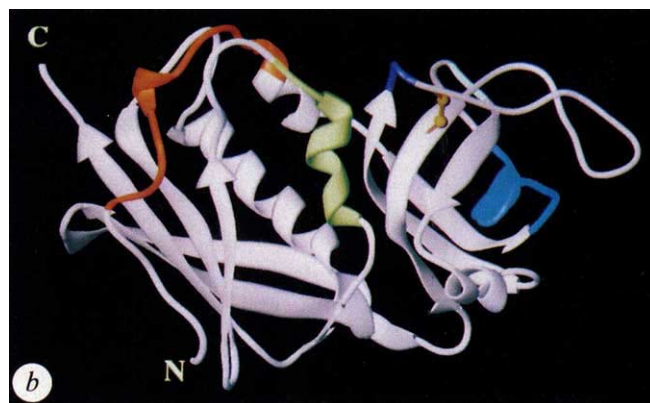


FIG. 2 a, A schematic secondary structure diagram of SEB. The two structural domains of the molecule are seen clearly. Cylinders and arrows represent α -helices and β -strands, respectively. The discontinuous line in the disulphide loop indicates the seven residues that could not be located in the electron density map. The residues forming the secondary structural elements are: 13–17 ($\alpha 1$), 21–29 ($\alpha 2$), 33–39 ($\beta 1$), 48–52 ($\beta 2$), 63–68 ($\beta 3$), 70–78 ($\alpha 3$), 81–89 ($\beta 4$), 112–120 ($\beta 5$), 127–138 ($\beta 6$), 141–151 ($\beta 7$), 154–156 ($\beta 8$), 157–172 ($\alpha 4$), 182–190 ($\beta 9$), 195–200 ($\beta 10$), 210–217



($\alpha 5$), 222–224 ($\beta 11$), 229–236 ($\beta 12$). b, A ribbon drawing showing the domain organization of SEB. Regions implicated by mutational analysis²⁵ are highlighted. Region 1 (residues 9–23), region 2 (residues 40–53) and region 3 (residues 60–61) are shown in red, blue and white, respectively. Residues 90–92 and 208–217 identified from the three-dimensional structure to be important for binding TCR are shown in dark blue and light green, respectively. The disulphide bond is shown in yellow.

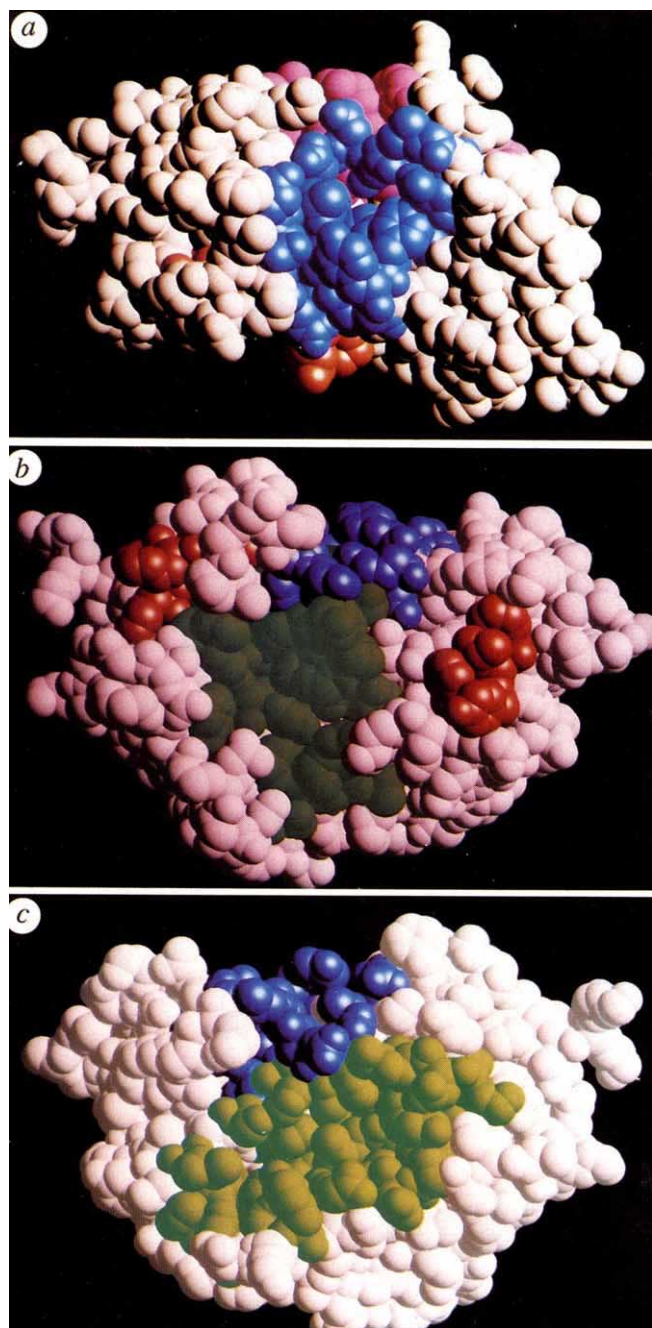
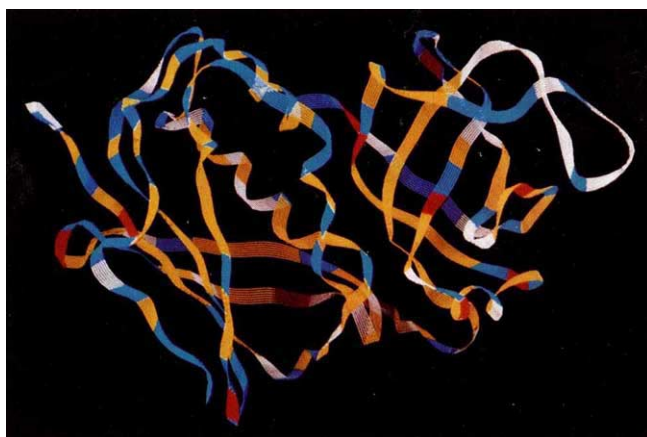


FIG. 4 A ribbon drawing showing the homology of SEB with other staphylococcal enterotoxin. Colour coding²: residues that are identical or that are changed to amino acids with similar properties at least among two of SEA, SED and SEE are in red. Residues that are identical or that are changed to amino acids of similar properties at least among two of SEB, SEC1 and SEC3 are in blue. Residues that are identical or that are changed to amino acids of similar properties at least among two of SEB, SEC1 and SEC3 and in at least two of SEA, SEE and SED are in yellow. The variable residues (shown in white) are either on turns, loops or in regions of the molecule exposed to the solvent, suggesting that the main chain folding pattern is similar for all staphylococcal enterotoxins.

context can then reveal information about the stereochemistry of the MHCII- and receptor-binding sites.

Region 1, defined as the stretch of residues from 9 to 23, is bifunctional as it affects both TCR and MHCII binding. Mutations in this region included residues in positions 9, 14, 17 and 23, as either a single or a double mutation. Residue 9 is located in an aperiodic region of the molecule between $\alpha 1$ and the N terminus. Asparagine at residue 23 (N23) is on the α -helix, $\alpha 2$, with the side chain pointing towards the solvent. It is the most important residue, being conserved among all staphylococcal enterotoxins and critical for TCR activation. Whereas only one of the five mutations at position 23 affected MHCII binding, all of them affected TCR activity. Mutations at residues 14 and 17 affected both MHCII binding and TCR activation. Residue S14 is on a very short α -helix ($\alpha 1$) and is exposed to the solvent whereas F17 is located at the other end of $\alpha 1$. The locations of D9, S14, F17 and N23 on the surface of the toxin are favourable for making critical contacts with MHCII and/or V β . Residue F17 points inwards and lies in a loop sandwiched between two other loops, of residues 174–179 and 203–209. This suggests that its replacement by serine (F17S) may have introduced structural changes which reduce MHCII binding and cytokine production.

FIG. 3 *a*, A space filling diagram looking into the TCR-binding site (shown in blue). The molecule is rotated 90 degrees top-forward about the horizontal axis from the orientation shown in Fig. 2*b*. Residues E22, N23, D29, D30, L58, N60, Y90 and Y91 are located along the rim and all of them except N60 and Y91 are pointing outwards from the hollow; the side chains of residues N60 and Y91 though exposed to the solvent are tilted towards the hollow. Although residues A87, N88 and L214 form the bottom of the binding site, the remaining residues form the walls of the cavity. The $\alpha 4$ and $\alpha 5$ grooves are shown in pink and brown, respectively. The TCR-binding site sits in the middle of and adjacent to these two grooves, which are on opposite sides of the molecule. *b*, A space filling diagram in the same orientation as Fig. 2*b*. The TCR-binding site is shown in blue. Residues in regions 1 and 2 implicated in MHCII binding²⁵ are shown in red. Residues in $\alpha 5$ and the groove associated with it are in green. The residues lining this groove are H12, **F17**, **M24**, **Y28**, **D161**, **R165**, E191, **F196**, W197, **Y198**, **D199**, **M201**, F208, K212, **Y213**, M215, **M216**, **Y217**, **D219**, **K221**, **V223** and **V228**. The residues conserved among all staphylococcal enterotoxin are in bold type. The other residues in this groove are conserved among SEB, SEC1 and SEC3. Residues in regions 1 and 2 and the $\alpha 5$ groove are on the same side of the molecule thus defining the likely MHCII-binding site. *c*, A space filling diagram showing the TCR-binding site in blue. Residues in $\alpha 4$ and the groove associated with it are in green. The molecule is rotated 180 degrees about the vertical axis from the orientation shown in Fig. 2*b*. The groove involving $\alpha 4$ is lined with residues D30, H32, S34, I36, **D83**, **F85**, **T119**, **H121**, Q125, Y129, **F146**, **D147**, **V148**, Q149, **T150**, N151, **K152**, **E159**, Y162, L163, H166 and **Y167**. The bold type indicates conserved residues. Of the remaining residues in the groove D30, S34, Q149 and Y162 are the same in SEB, SEC1, and SEC3, whereas the rest are variable.



The association of S14 (on $\alpha 1$) with MHCII binding, and N23 (on $\alpha 2$) with TCR activity, reveals the structural basis underlying the bifunctional role of region 1. Although the region consists of a small number of sequential amino acids, they are distributed on separated but adjacent elements of the secondary structure that are engaged in different functions. The proximity of $\alpha 1$ and $\alpha 2$ is consistent with the suggestion²⁶ that the amino acids in region 1 are situated in the trimolecular complex near the junction of V β and MHCII.

Region 2 is defined as residues 40 to 53 and was suggested to be important in all staphylococcal enterotoxins in mediating binding to MHCII. About half of the mutations in this region involved the conserved residue F44. Other mutations involved residues 41, 45, 46, 48, 52 and 53. These changes affected MHCII binding and consequently the TCR activation. Thus, this region is probably specific for MHCII binding. Residues 48–52 are in β -strand $\beta 2$. Residue F44 is on a turn connecting $\beta 1$ and $\beta 2$ with the side chain exposed to solvent. It is situated favourably for engaging in critical hydrophobic binding contacts with MHCII.

Region 3 is made up of two residues, 60 and 61, and mutation of either one affects the TCR activation but not MHCII binding. Residues 60 and 61 are in the loop connecting $\beta 2$ and $\beta 3$ and are exposed to solvent.

TCR binding site

There is a shallow cavity in the molecular surface, that is formed by residues from both domains, namely E22, N23, V26, L27, D29, D30, N31, V33, D55, L58, N60, Y61, A87, N88, Y89, Y90, Y91, Q92, T112, Q210 and L214 (Fig. 3a). Residues in bold type are conserved in all staphylococcal enterotoxins. Residues N31 and A87 are the variable residues and the rest are conserved in SEB, SEC1 and SEC3. Whereas residues N23, N60 and Y61 of regions 1 and 3 form a part of this cavity, residues from region 2 do not. Thus, residues in this cavity are important for TCR activation but not for MHCII binding. The molecular topology, as well as the mutational evidence, clearly indicates that this is the TCR-binding site. It is interesting to note that residues 90–93 initially formed a fourth region in the mutation study²⁶, but were eliminated from their analysis because they believed that mutations in this region might affect the stability of the molecule. From the three-dimensional disposition of these residues it appears that they too are important for TCR activation. In addition, the spatial arrangement of residues in the region 208–217 indicates that they may also be important for TCR activation. Our predictions were later confirmed by mutational analysis showing that residues corresponding to 208 and 209 of SEB are specific for TCR activity in SEA (this issue⁴⁵). Residues 208 and 209 are in the outer rim of the TCR site in SEB. Also, in another related toxin²⁷, the residue corresponding to Y175 (ref. 3) in SEB has been found to be critical for mitogenic activity. This residue is close to the TCR site in SEB and may define the outer rim of the site. The three adjacent loops (Fig. 2b) taken collectively as a characteristic structural unit may be functionally important.

MHCII binding site

Unlike the TCR-binding site, the MHCII-binding site is not obviously identified by the topology and mutational studies. This is because residues implicated in MHCII binding are separated in the molecule, and do not coalesce into a single site though they are on the same side of the molecule. The available mutational data indicate that MHCII binding occurs on the $\alpha 5$ face of SEB. The groove on the $\alpha 5$ side is well formed and runs along the length of the helix. This groove and residues 13–17 and 44–52 implicated in MHCII binding²⁶ are situated on the same side of the molecule (Fig. 3b). This groove and the TCR-binding site are adjacent to one another, forming two sides of a 'wedge', making it possible for TCR and MHCII to be in close proximity²¹. In addition, residue H187 in SEA interacts with

H81 of MHCII through a zinc bridge (J. D. Fraser, personal communication). Residue I190 of SEB, corresponding to H187 of SEA, is situated on the rim of this groove. This evidence strongly suggests that this is the MHCII-binding site.

Possible role of the $\alpha 4$ groove

The groove involving $\alpha 4$ (Fig. 3c) lies on the side opposite the suggested MHCII-binding site. It contains residues 113–166 of SEB, which are homologous to the human and mouse invariant chain³. Invariant chain is associated with nascent MHCII molecules and supposedly prevents the binding of peptides produced endogenously in the cell³. As the MHCII molecule reaches the surface of the cell, the invariant chain separates and promotes the binding of peptides. The sequence homology of the invariant chain to the staphylococcal enterotoxins suggests that this groove may have a role in the binding of superantigens to MHCII. But it has been observed that the binding of superantigens to MHCII does not prevent antigenic peptides from binding as well, and, to our knowledge, residues 113–166 have not been implicated in MHCII binding experimentally.

The active site for emesis is thought²⁸ to be the region corresponding to residues 113–126. Of these 14 residues, 11 are conserved in all staphylococcal enterotoxins. In particular, H121 is thought to be the most important residue. It is in the stretch connecting domains 1 and 2, and is exposed to the solvent. Three of the five histidine residues in SEB, H32, H121 and H166 are situated in the $\alpha 4$ groove. As exhaustive carboxymethylation of histidines is known to prevent all activities^{29,30} in staphylococcal enterotoxins, this groove may be of biological importance.

Conclusion

The three-dimensional structure of SEB shows that the superantigenic and the putative emetic²⁸ sites may be different. The two grooves involving $\alpha 4$ and $\alpha 5$ are on either side of and adjacent to the TCR-binding site (Fig. 3a). The sequence homology in staphylococcal enterotoxins and the location of variable residues in SEB (Fig. 4) suggest that the SEB-folding pattern may be common to all staphylococcal enterotoxins. The organization of the sites for biological activity deduced from this structural analysis should aid understanding of the mechanism of superantigenicity in staphylococcal enterotoxins and in designing drugs and vaccines to counteract their pathological effects. □

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LETTERS TO NATURE

Rates of collapse and evaporation of globular clusters

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OF the known galactic globular clusters, ~140 in number, about one-fifth have undergone core collapse, and an unknown number have evaporated altogether. From observational estimates of the dynamical relaxation times of globular clusters, measured at their cores and at their half-light radii, we estimate here the present rates at which core collapse and evaporation are occurring. We find a core collapse rate of 2 ± 1 per Gyr, which for a galactic age of ~12 Gyr agrees well with the fact that 27 clusters have surface brightness profiles with the morphology expected for the post-collapse phase. We find a destruction and evaporation rate of 5 ± 3 per Gyr, suggesting that a significant fraction of the Galaxy's original complement of globular clusters have perished, through the combined effects of mechanisms such as relaxation-driven evaporation and shocking due to interaction with the galactic disk and bulge.

Recent progress in understanding globular clusters and their dynamical evolution (for reviews see refs 1, 2) has been made both by studying individual clusters in detail³ and by studying the statistical properties of the population of galactic globular clusters⁴. Here we adopt the latter approach, focusing on relaxation times.

Several globular clusters seem to be on the brink of core collapse, in the sense that their predicted collapse time is much smaller than their current age⁵. Unless we happen to live at a special time just before the clusters collapse, many of them have already undergone core collapse⁶—if so, where are they? The most straightforward answer is to identify them with a subpopulation of those clusters that have short enough central relaxation times t_{rc} (ref. 7). Increasingly detailed observation shows that ~20% of the globular clusters have a surface brightness profile close to a power law with slope ~-1 near their centres, making them prime candidates for identification as post-core-collapse clusters^{8,9}.

Core collapse and the possibility of subsequent core oscillations^{10–12} are interesting phenomena, but they do not threaten the existence of a globular cluster, as they only affect the inner few per cent of the stars. There are, however, several processes that tend to destroy globular clusters. One of these is two-body relaxation, which causes globular clusters to evaporate by replenishing the high-energy tail of the maxwellian distribution. As a result, stars escape through the tidal boundary, and consequently all globular clusters will eventually evaporate. Other processes, such as disk shocking and bulge shocking, can greatly

speed up this destruction process^{4,13}. Here we will not attempt to discriminate between these processes, simply grouping them under the general term of evaporation. In particular, we neglect the process of dynamical friction, which is a significant destruction channel only for those globular clusters closest to the centre of the Galaxy^{13,14}.

We use recent observations of structural parameters of globular clusters to calculate central and half-light relaxation times. From these, we derive the current rates of core collapse and evaporation of globular clusters, together with an estimate of the uncertainties involved in this derivation. To calculate the relaxation times, we use the structural parameters tabulated in ref. 4, with minor updates¹⁵, supplemented by data on cluster half-light radii from ref. 16. This allows us to compute the central relaxation times, t_{rc} , for 103 clusters with the King¹⁷ model morphology (KM), and for an additional 27 clusters with a post-core-collapse (PCC) morphology^{8,9}. It also allows us to compute the half-light relaxation times, t_{rh} , for 98 clusters. From error propagation of the input quantities, we estimate the uncertainty of the relaxation times to be about a factor or two.

The central relaxation times were computed following Spitzer¹⁸ (his equation 2-61, transformed using the virial theorem) as

$$t_{rc} = 8.34 \times 10^6 \rho_0^{1/2} m_*^{-1} r_c^3 \ln^{-1}(0.4N)$$

where t_{rc} is in years, $\rho = M_{cl}/(\mu r_c^3)$ is the central density in solar masses ($M_\odot$) per cubic parsec, computed from the total cluster mass M_{cl} , the core radius r_c in parsec and the fractional core mass μ (see Table II of ref. 17), m_* is the average stellar mass in $M_\odot$ and N is the number of stars in the cluster. We assumed $m_* = 1/3$, and evaluated M_{cl} from the total absolute V-band magnitudes by assuming $(M/L_V) = 3$. The half-light relaxation times were computed following equation 2-63 of ref. 18 as

$$t_{rh} = 2.06 \times 10^6 M_{cl}^{1/2} m_*^{-1} r_h^{3/2} \ln^{-1}(0.4N)$$

where t_{rh} is in years and r_h is the half-projected-light radius in parsec. We note that t_{rc} is not defined for PCC clusters, but t_{rh} is. An upper bound for t_{rc} may be ~10⁷ yr, and a reasonable lower bound the crossing time for a 0.1-pc core, which is ~10⁴ yr.

Figure 1 shows the distributions of cluster relaxation times. The distributions of t_{rc} (Fig. 1a) and t_{rh} (Fig. 1b) are strikingly different. Whereas the distribution of t_{rc} is well represented by a power law with a slope of about -1.2 (except at the low- t_{rc} end), the distribution of t_{rh} seems to have a well defined maximum around 0.5 Gyr, with a steep falloff (power law slope ~-2.7) at longer t_{rh} ; the data are also consistent, however, with a flat distribution at times less than ~1–2 Gyr. There may be a flattening in the t_{rc} distribution below ~2 × 10⁷ yr. We represent the PCC clusters in Fig. 1a by dotted horizontal lines whose length represents a possible bin width. Regardless of what lower limit is chosen for their t_{rc} , they clearly form an 'excess' at the low- t_{rc} end. Moreover, it is possible that some of the short- t_{rc} , KM-like clusters are unrecognized and misclassified PCC clusters, which would both increase the 'PCC excess' and emphasize the flattening of the t_{rc} distribution at the low end.

A power-law fit to the t_{rc} data has a slope of -1.04 ± 0.08 ; omitting the first one or two points (as there may be a flattening

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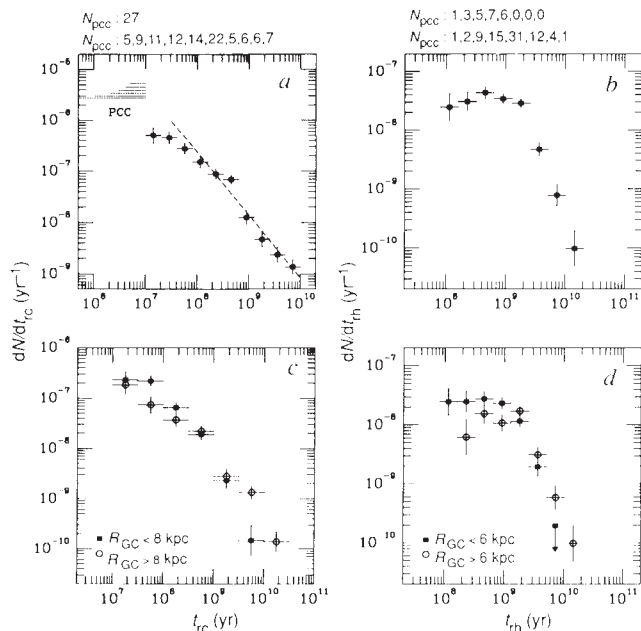


FIG. 1 *a*, Distribution of central relaxation times (t_{rc}) for a set of 103 galactic globular clusters. The 27 PCC clusters occupy a bin whose height depends on the assumed bin width, as indicated by the dotted lines. *b*, Distribution of projected-half-light relaxation times (t_{rh}) for a set of 98 galactic globular clusters, including 20 clusters with a PCC morphology. *c*, The t_{rc} distributions for the data subsets divided near the median galactocentric radius (8 kpc). *d*, The t_{rh} distributions for the data subsets divided near the median galactocentric radius (6 kpc; the PCC clusters, which tend to have lower galactocentric radius, are included in *b* and *d* but not *a* and *c*). In all four panels, the horizontal bars indicate the bin widths, and the vertical bars the poissonian errors. Numbers of clusters in subsequent bins are listed above *a* and *b*.

of the distribution for low t_{rc} , we get slopes of -1.14 ± 0.08 or -1.22 ± 0.10 , respectively. There may be a break in the distribution at $t_{rc} \approx 6 \times 10^8 \text{ yr}$, dividing the distribution in two segments, both with power-law slopes very close to -1 . This feature is significant at about a 2σ level, and we will not discuss it further. Better data are needed to check on its reality and possible physical significance.

The observed distributions of t_{rc} and t_{rh} may be affected by data selection and external dynamical evolution effects, and we must consider those before attempting to deduce something about the initial conditions or internal dynamical evolution effects.

By definition, t_{rc} is well correlated with r_c (Pearson and Spearman correlation coefficients $p = 0.992$ and $s = 0.974$) and, through the fractional mass μ , with the King concentration parameter c ($p = -0.937$, $s = -0.867$). Both r_c and c are in turn correlated with the distance to the Galactic Centre, R_{GC} , and the galactic plane, Z_{GP} (correlation coefficients ~ 0.5), in the sense that more concentrated and/or small-core clusters, which therefore have short t_{rc} , are found closer to the Galactic Centre or plane (see also refs 4, 19). Indeed, t_{rc} correlates directly with R_{GC} ($p = 0.548$, $s = 0.562$) and Z_{GP} ($p = 0.517$, $s = 0.552$). A similar situation exists for t_{rh} . There are two consequences of these correlations. First, short- t_{rc} and PCC clusters are more likely to be obscured, and the selection effects would depress the observed t_{rc} distribution at the low end. Second, tidal destruction by shocks from the disk or bulge passages will be more efficient in destroying clusters with long t_{rc} or t_{rh} , thus depressing the long- t_{rc} (or t_{rh}) ends of the distributions.

To test this, we plot in Fig. 1 *c* and *d* the distributions divided by the median R_{GC} . As expected, the inner distributions have steeper cutoffs. The differences between the inner and the outer distributions are probably illustrative of the magnitude of external dynamical evolution effects, such as tidal shocks and increased evaporation through the stronger galactic tides.

The current rate at which globular clusters undergo core collapse and evaporate can be estimated from the observational data provided in the previous section, using the procedure outlined in ref. 7. Let us denote the distribution function of globular cluster relaxation times by $f_i(t_{ri}) = dN/dt_{ri}$ with i standing for c or h , to indicate the central relaxation time t_{rc} or the half-light relaxation time t_{rh} , respectively (our f corresponds to n in ref. 7).

The distribution function is time-dependent, and we can only observe the present $f_i(t_{ri}, T)$ with T the current age of the

globular cluster system. We assume a power law for the initial distributions $f_i(t_{ri}, 0) = A_i t_{ri}^{\alpha_i}$. These power laws can still be recognized on the right-hand sides of Fig. 1 *a* and *b*. At the left-hand sides, the distribution functions flatten off as a result of dynamical evolution. For central relaxation times, this follows from the fact that the rate of change $B_i = dt_{ri}/dt$ is independent of time, as a result of the dynamics of core collapse⁷. For the half-mass relaxation times, similar behaviour can be derived from the dynamics of the evaporation through two-body relaxation in a cluster limited by galactic tides. We will denote the level at which the flattening occurs by $C_i = f_i(0, T)$.

We thus arrive at the following recipe. We first choose a value for the clusters' age T , then determine A_i , α_i and C_i from a fit to the data in Fig. 1. The rate of change of relaxation times for an individual cluster then follows as

$$B_i = \frac{1}{T} \left(\frac{C_i}{A_i} \right)^{1/\alpha_i}$$

This gives us for the current rates R_c of core collapse and R_h of evaporation:

$$R_i = B_i C_i = T^{-1} A_i^{-1/\alpha_i} C_i^{1+1/\alpha_i}$$

The data in Fig. 1 are too sparse to warrant a formal fit. We have estimated by eye the following parameter values, together with our guesses for the rough uncertainties: $A_c = 15 \pm 7$, $A_h = 150 \pm 100$; $\alpha_c = -1.2 \pm 0.1$, $\alpha_h = -2.7 \pm 0.1$; $C_c = 600 \pm 300$, $C_h = 40 \pm 15$. All numbers are in units of Gyr, or Gyr^{-1} as applicable. We use a cluster age $T = 12 \pm 2 \text{ Gyr}$. With these fits, we find the following core collapse and cluster evaporation rates: $R_c = (2 \pm 1) \text{ Gyr}^{-1}$, $R_h = (5 \pm 3) \text{ Gyr}^{-1}$.

The fact that we can obtain these rate numbers indicates the progress made in both theory and observation of globular clusters. A decade ago, the observations were not accurate enough to derive these numbers, and our theoretical understanding of core collapse was insufficiently developed to interpret the data available at the time.

In our derivation of the current rates of core collapse and evaporation, we have treated the ensemble of globular clusters as if it were made up of a set of identical clusters. This is an approximation, and in the end is justified mainly observationally, by the reasonable resemblance of the data in Fig. 1 to the functional form discussed in ref. 7. Some of the many factors that would modify our simple analysis are differences in mass spectrum between different clusters (affecting the rate of core collapse), and differences in orbits around the Galactic Centre (affecting the rate of evaporation). The latter are likely to be important, as tidal disk and bulge shocking depend strongly on galactocentric distance and have an important role in the

evolution of globular clusters^{13,14}. In the light of these theoretical uncertainties, we feel that our simple power-law approach is a good way to obtain a crude overall measure for the evaporation rate. Any more accurate fit to the data would involve a modelling effort far beyond our scope here. □

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Resolution of the circumstellar gas around the Be star ψ Persei

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Be STARS are rapidly rotating, non-supergiant B stars showing hydrogen-line emission and characterized by an excess of continuum emission from near-infrared to radio wavelengths. The latter is thought to arise from thermal bremsstrahlung in hot circumstellar gas¹, for which both spherical and disk-like geometries have been proposed^{2,3} to explain observations at different wavelengths. The first Be star to be detected at radio wavelengths was the B5 giant ψ Persei^{4–6}, which is the brightest radio-emitting Be star. Using the Very Large Array, we have resolved the circumstellar envelope of ψ Per at 15 GHz, and find that the radio emission comes from a non-spherical distribution of thermally radiating gas. The radio-emitting region has a major axis of 111 ± 16 mas (17 AU at a distance of 155 pc), and is unresolved, with a 3σ upper limit of 68 mas, along its minor axis. Our observations confirm the proposal, due to Struve in 1931 (ref. 5), of equatorially enhanced circumstellar plasma distributions as the source of Be star emission.

We observed ψ Per on 16 August 1991 at 15 GHz with the highest-resolution configuration of the Very Large Array (VLA), giving a synthesized beam of 176×144 mas. The complex gains of the 26-antenna array were monitored by frequent observation of the nearby bright source 3C84, and the absolute flux calibration was established by an observation of 3C48. The total integration time was ~ 11 hours, giving a 1σ noise level on the synthesized image of $40 \mu\text{Jy}$. The final image is shown in Fig. 1. A two-dimensional gaussian fit to the image of ψ Per indicates a source with an angular size of 132×83 mas with a position angle of 152° , that is, a resolved source which is not symmetric on the

plane of the sky.

When the source size is close to that of the telescope beam, as in this case, more accurate estimates of the source dimensions can be obtained from analysis of the interferometer data before imaging. An image derived from observations taken with an interferometric array is the result of Fourier transformation of the two-dimensional complex visibility data, which depend on the baseline length of the array. For an unresolved source (a point source), at the phase centre of an interferometer array, the real amplitude of the complex visibility is independent of baseline length, and equal to the total flux density of the source.

To confirm that ψ Per is resolved, we examined the complex visibility data directly. After phase-centring, the real visibility amplitudes of ψ Per were calculated as a function of baseline length (Fig. 2). The data points in Fig. 2 are the means and errors of the visibility amplitude from a total of almost 5×10^5 visibility points which have been binned into 15 baseline length ranges. The increasing uncertainty with baseline length is the result of a decreasing number of visibility data points at longer baselines. The solid line represents a circular gaussian fit to the data and shows that over the baseline range of the VLA at 15 GHz, the real visibility amplitude of ψ Per decreases by $\sim 75\%$. The emission is well resolved by the array. The fit gives a total flux density of $804 \pm 60 \mu\text{Jy}$ and a mean angular size of 74 ± 13 mas. The real amplitude of the calibration source 3C84 is also shown in Fig. 2 for direct comparison. It decreases by less than 1% over the baseline range. We conclude, therefore, that the decrease in visibility amplitude of ψ Per at long baselines is a resolution effect and not due to an error in calibration of the complex antenna gains or de-correlation caused by atmospheric effects.

To determine the shape of the radio brightness distribution of ψ Per on the plane of the sky, we examined the two-dimensional distribution of the real visibility data. The visibility data were binned into a 30×30 grid. A two-dimensional gaussian fit to the gridded data gives a source that has a total flux density of $837 \pm 60 \mu\text{Jy}$ and a major axis diameter of 111 ± 16 mas. The source is unresolved in the minor axis direction. The position angle of the major axis of the Gaussian is $158 \pm 10^\circ$. This result agrees well with the source parameters determined from the

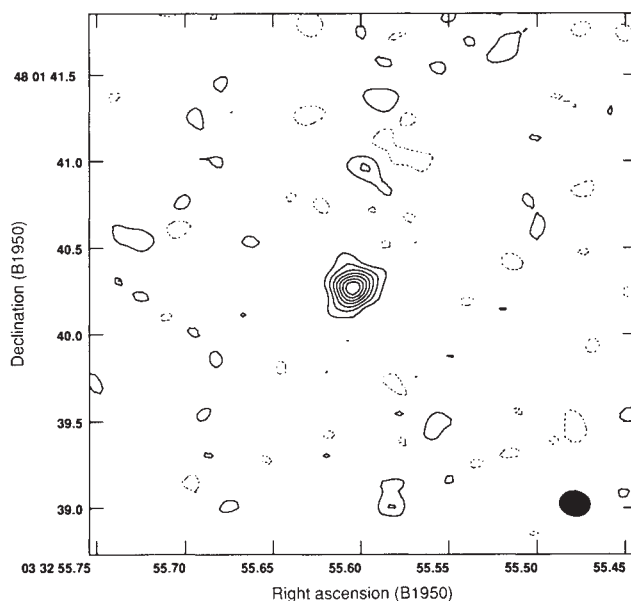


FIG. 1 Image at 15 GHz of ψ Persei. Contour levels are set to $-6, -4, -2, 2, 4, 6, 8, 10, 12, 14\sigma$, where 1σ is $40 \mu\text{Jy}$. The full width at half maximum of the beam is shown at the lower right-hand corner.

gaussian fit to the image in Fig. 1. Clearly, the radio-emitting region around ψ Per is not symmetric on the plane of the sky.

The minimum angular size that can be measured by this fitting method was determined using a Monte Carlo simulation method. Two-dimensional gaussians with fixed major axis size of 111 mas and a range of minor axes were added to a noise surface to simulate visibility data. The fitting procedure then determined the angular size of the minor axis and its uncertainty from the simulated data. Comparison of the measured angular size with the ratio of measured size and its uncertainty (the signal-to-noise ratio of the size) gave an angular size of 68 mas for a signal-to-noise ratio of 3. This was taken to be the 3σ upper limit to the angular size of the minor axis.

Debate about the geometry of the circumstellar envelopes around Be stars has arisen partly because the observations at different wavelength regions have been interpreted to indicate apparently conflicting characteristics of the plasma. At ultraviolet wavelengths, absorption-line profiles of highly ionized metals such as Si IV and C V suggest the existence of plasma with very high velocity ($\sim 1,000 \text{ km s}^{-1}$) and low density⁸. But optical emission-line strengths and the level of excess continuum emission from near-infrared to radio wavelengths indicate the presence of low-velocity (less than 20 km s^{-1}), high-density plasma⁹. To account for these observations, the circumstellar gas must contain regions of high and low velocity. A spherically symmetric circumstellar gas distribution would require decelerated or accelerated gas³.

The 15-GHz image of ψ Per demonstrates that a spherically symmetric circumstellar gas distribution is not applicable to this star. The spectral line shapes of ψ Per at ultraviolet and visual wavelengths¹⁰ imply that there is relatively high-density circumstellar gas along the line of sight to the star. Thus, if the circumstellar gas is in the form of a disk, the plane of the disk is closely perpendicular to the plane of the sky. The projected rotational velocity of ψ Per is high ($v \sin i \approx 300 \text{ km s}^{-1}$; ref. 11) indicating that the rotation axis of the star lies closely in the plane of the sky, as expected if the plane of the circumstellar disk is in the equatorial plane of the star.

The suggestion that circumstellar gas in Be stars is equatorially enhanced was first proposed by Struve in 1931. Such a circumstellar gas distribution in Be stars has been inferred from a number of observations. Most notably, polarization observations reveal a considerable degree of linear polarization in the optical and near-infrared continuum of many Be stars¹²⁻¹⁴. This is interpreted as arising from scattering from free electrons whose density is higher in the equatorial plane of the star. Linear polarization observations of ψ Per^{13,14} show a polarization posi-

tion angle of $\sim 45^\circ$. For an asymmetric electron distribution, maximum polarization occurs perpendicular to the major axis of the distribution. The observations therefore indicate an asymmetric electron distribution around ψ Per with a position angle of $\sim 135^\circ$. This is in good agreement with the position angle derived from the radio observation.

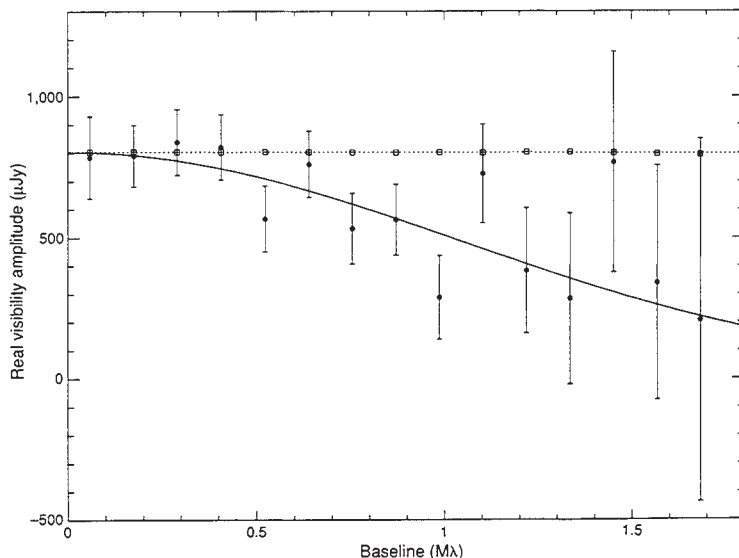
The angular size of the source gives a lower limit to the brightness temperature of 870 K. We can only give a lower limit to the brightness temperature as the minor axis dimension is an upper limit. The brightness temperature of a source emitting because of free-free processes is a lower limit to the kinetic temperature of the source. These processes arise in plasma that has been photoionized by Lyman continuum photons. For typical cooling mechanisms in photoionized gas of solar composition we expect kinetic temperatures of $\sim 10^4 \text{ K}$. A temperature of 870 K is unrealistically low for the kinetic temperature of the circumstellar plasma. A brightness temperature of 5,000 K would imply a minor axis size of $\sim 12 \text{ mas}$, which suggests that the circumstellar plasma distribution around ψ Per is highly flattened, with a major/minor axis ratio ≥ 10 .

The detection of centimetre-wavelength radio emission from Be stars^{4,5} demonstrates that the high-density plasma extends, in some cases at least, to very large radii. If we assume that the major axis of the gaussian fit to the data represents the diameter of the optically thick emitting region, we can attribute a linear size to the radio-emitting region. For a distance to ψ Per of 155 pc (ref. 15), the major axis dimension is 3,700 solar radii (17 AU), a dimension comparable to the size of the Solar System. This value is roughly four times the minimum diameter calculated from radio flux density observations⁵. The increase in the size of the radio-emitting region is not unexpected, because the calculation of minimum size assumed a totally optically thick source and the radio spectral index of ψ Per indicates that the radiating plasma is partially optically thin at radio frequencies⁶.

Analysis of the continuum spectra of Be stars^{1,16} suggests that the circumstellar material is flowing outwards. It is important to determine whether the circumstellar material is lost from the star. For mass loss to occur, the outflow velocity must at some point exceed the escape velocity. The escape velocity at a distance of $3,700 R_\odot$ from a star of mass 5 solar masses¹⁷ is 23 km s^{-1} . Observations of O I and Fe III shell lines show velocity dispersions of $\sim 30 \text{ km s}^{-1}$ in the high-density gas around ψ Per¹⁰. For a kinetic temperature of $\sim 10^4 \text{ K}$, the thermal velocity is $\sim 15 \text{ km s}^{-1}$. It thus seems likely that the outward velocity at $3,700 R_\odot$ is at least of the same order as the escape velocity.

The physical dimension of the circumstellar matter may be larger than $3,700 R_\odot$. In the case of outflow with a radial density

FIG. 2 Real visibility amplitude of ψ Persei (●) and 3C84 (□) at 15 GHz, as a function of baseline length in units of 10^6 wavelengths. The data for 3C84 have been normalized to the zero baseline amplitude of ψ Per. The solid line represents a circular gaussian fit to the ψ Per visibilities with fit parameters of $804 \pm 64 \mu\text{Jy}$ total flux density and an angular size of $74 \pm 13 \text{ mas}$. The dashed line is a circular gaussian fit to 3C84, which gives a point source with a flux density of 33.9 Jy. The error bars for 3C84 are smaller than the symbols.



distribution of the form $\rho \propto r^{-\beta}$ (ρ is density, r radius), the measured angular size of the emitting region is a function of the observing frequency and the radial density gradient, namely $\theta \propto \nu^{2/1-2\beta}$, where θ is the angular size of the radio 'photosphere' at frequency ν . If the circumstellar gas around ψ Per is an outflow of this form, we have measured the size of the radio 'photosphere' at 15 GHz. An observation at a lower frequency should yield a larger radio photosphere. $\square$

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CHON as a component of dust from comet Halley

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AN important discovery made by the 1986 spacecraft encounters with comet Halley was that of 'CHON particles', dust that is predominantly composed of the light elements carbon, hydrogen, oxygen and nitrogen^{1–3}. Using three sets of particle mass spectra that were critically selected for high dynamic range and a minimum of defects, we investigate here the relationship between CHON and silicate materials. We find that there are essentially no pure CHON particles in the 0.1–1 μ m size range sampled; instead, essentially all Halley particles sampled by the mass spectrometers are a mixture of both CHON and silicate components. The earlier evidence for pure CHON particles^{1,4,5} came from mass spectra with low dynamic range, in which only the highest major-element peaks could be detected. Our data show that the CHON and silicate components are interdispersed at submicrometre scales, and there is evidence that sublimation of volatile organic material occurs, bringing many particles to a common proportion of CHON and silicate material.

The particle composition data for this study, obtained by the PUMA-1 and PIA mass spectrometers flown on the Vega-1 and Giotto spacecraft during their encounters with Halley⁶, consist of time-of-flight mass spectra produced by the impact of individual micrometre and submicrometre dust particles on metal targets. Every twenty-fifth spectrum was sent in an uncompressed, time-recorder format with high-frequency sampling. For the remaining spectra, onboard data compression removed all information about mass peaks except their height and location in time. It seems that PIA lost two of its nominal five

decades of dynamic range, and only the most abundant elements are visible in typical mass spectra. Because data compression removed further details of the spectra, we have used only uncompressed spectra from PIA. The PUMA-1 instrument operated as expected, and its spectra have high dynamic range and sensitivity. On PUMA-1 the ion acceleration voltage was toggled every 30 s to create narrow and wide acceptance windows (also termed 'long' and 'short'^{7,8}) for the ions' initial energies: ~ 0 –50 eV and ~ 0 –150 eV, respectively. The narrow energy spectra suffer from a heavy suppression of light elements^{7,8}, and we have used only the wide spectra.

The method used to select particle mass spectra for analysis is extremely important, because their quality varies. We used a selection procedure⁹ that rejected spectra with excessive noise, insufficient major-element signals, molecular ions or terrestrial contaminants. Other studies^{8,10–13} have generally been less discriminating. Our selected spectra are nearly free of contamination by molecular species and are otherwise of high quality, making element identifications unambiguous and accurate. Our automated selection procedure reduces the likelihood of human bias. To guard against spurious peaks in our selected spectra, we set a detection threshold of five amplitude units out of the total range of 10^5 and required that each peak stand above its local background by at least a factor of three. Our procedure is therefore slightly biased against smaller dust particles. Characteristics of the resulting data sets A, B and C are shown in Table 1. These samples form 9%, 14% and 1% of their respective types of spectra. The proportionally small size of set C is due to the paucity of identifiable peaks in PIA spectra. Ion-proportional data used to produce the table and figures are based on peak heights for set A and integrated area under the peaks for sets B and C. Our analysis shows that peak height (the only information recorded in the compressed modes) is proportional to the integrated area under a peak (indicating the total number of ions).

Carbon is generally a strong, unambiguously distinguishable peak, whereas oxygen, probably because it forms undetected negative ions, has a variable ion yield. Even if it gave a reliable mass peak, oxygen would not be a sensitive indicator for distinguishing between CHON and silicate components. Hydrogen is difficult to interpret because it is often accompanied by noise; there may also be H contamination in the target. In Halley's dust $N/C \approx 0.03$ –0.07, implying that N is a minor constituent of CHON material^{14,15}. Moreover, in the compressed-mode spectra N is difficult to resolve from C and O. Mg, Si, Ca and Fe are all easy to resolve and interpret, and all are likely to occur in silicates, although Fe probably occurs in sulphides as well^{7,9,10,16,17}. We have therefore taken C ion counts to represent the CHON component, and the sum of the ion counts of Mg, Si, Ca and Fe to represent the 'rock' component.

Our bulk ion ratios (ratios of the sums of ion counts from all particles) $\sum C/\sum Si$, $\sum Mg/\sum Si$, $\sum Fe/\sum Si$ and $\sum C/(\sum Mg + Si + Ca + Fe)$ match published estimates¹⁰ of the bulk atom ratios for Halley dust to within a factor of four (see Table 1). The $\sum Ca/\sum Si$ ratio does not match well, but Ca is a less prevalent element. Despite the possibility of unknown instrumental effects that create variable ion yields, in what follows we shall assume that ion counts are roughly proportional to atoms in the pre-impact dust particle. A useful rough measure to compare CHON with 'rock' composition is thus

$$C/R \equiv C/(Mg + Si + Ca + Fe)_{\text{ion}} \propto C/(Mg + Si + Ca + Fe)_{\text{atom}} \\ \propto \text{CHON}/\text{'rock'}_{\text{atom}}$$

Carbon and $(Mg + Si + Ca + Fe)$ are compared in Figs 1 and 2. The histograms of C/R for the three data sets show similar sharp peaks. C/R varies over five orders of magnitude, but the lack of a break in the distribution indicates that the particles do not consist of separate CHON and 'rock' particle families. The large number of particles in data set A shows a strong

TABLE 1 Dust composition from three data sets

		Element occurrence (%)*						Bulk ratio ($\sum \text{element}/\sum \text{Si}$) _{ion}				$\sum C_{\text{ion}}$
Type of mass spectra		Number of particles	C	Mg	Si	Fe	Mg or Si or Fe	C	Mg	Ca	Fe	$\sum (\text{Mg} + \text{Si} + \text{Ca} + \text{Fe})_{\text{ion}}$
Set A	PUMA-1, compressed, wide energy window	280	93	88	94	68	99	7.2	1.5	0.12	0.43	2.3
Set B	PUMA-1, uncompressed, wide energy window	17	100	94	94	76	100	4.0	1.5	0.28	0.35	1.3
Set C	PIA, uncompressed	35	69	86	83	57	94	15.	1.4	0.019	0.49	5.3 [†]
All sets	—	332	91	88	92	67	98	‡	‡	‡	‡	‡
Atom ratios from ref. 10§		—	—	—	—	—	—	4.4	0.54	0.034	0.28	2.4

Comparisons of major element composition of Halley dust particles, as obtained from three mass spectrometer data sets. Uncertainties of the ratios shown in the last five columns are $\pm 19\%$.

^{*} Percentage of particle set containing measurable C, Mg, Si, Fe or at least one of Mg, Si and Fe.

[†] Carbon in this data set is dominated by the largest particle. Removing it reduces this ratio to 1.6.

[‡] Units of the three data sets are not directly comparable.

[§] Converted from ratios with respect to Mg in ref. 10.

concentration near $C/R \approx 1$ (50% of the particles have C/R between 0.4 and 4.0), producing the strong peak in Fig. 2a.

Table 1 shows for each data set the fraction of particles that contain one or more of the elements of interest. Evidently few particles, from the largest to the smallest, are either entirely organic or entirely inorganic: only 2% of the particles in all three data sets lack detectable Mg, Si or Fe. Furthermore, with the exception of a few possible iron-nickel sulphide particles, we have found no particles that appear to be all one mineral grain. If the dust particles sampled by PUMA-1 and PIA are typically $\leq 1 \mu\text{m}$ in size^{12,18,19}, then finely divided CHON and non-CHON materials must both occur at size scales down to a few hundredths of a micrometre. This result is consistent with the small (0.01–0.1 μm) mineral grains and organic components seen in the matrix material of porous chondritic interplanetary dust particles^{20–22}.

The distinctions that were earlier drawn between CHON dust

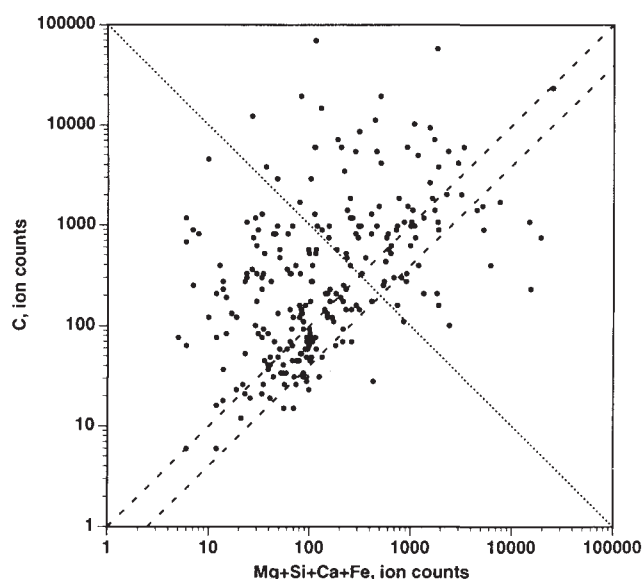


FIG. 1 Carbon against principal rock-forming elements in Halley's dust for data set A (PUMA-1 compressed modes, wide energy window). Ion counts of carbon are plotted against the sum of ion counts of Mg, Si, Ca and Fe. Ion counts shown are assumed to be roughly proportional to actual atoms in the dust particles. Ratios $C/(\text{Mg} + \text{Si} + \text{Ca} + \text{Fe}) = 1$ and 0.4 are indicated for reference by upper and lower dashed lines, respectively. Systematic errors are due primarily to quantization and amount to roughly $\pm 19\%$ in the abscissa and $\pm 9\%$ in the ordinate. The dotted line is an arbitrary subdivision into 'large' and 'small' particles used to produce Fig. 2d and e.

particles and non-CHON particles and the impression that there were numerous all-CHON particles^{1,4,5} seem to have been influenced by PIA data, which lost two decades of dynamic range and would not have shown the nearly universal occurrence of the rock-forming elements. The CHON material is merely one, albeit ubiquitous, component of the dust. Silicate minerals or glasses or iron-nickel sulphides^{9,23} are likewise present in nearly all particles. Pure CHON and pure silicate particle types probably do not exist at the particle size range sampled.

The lack of resolution of pure CHON and non-CHON components in Fig. 1 suggests further that the two components are mixed on a very fine scale. The wide dispersion of compositions provides constraints on the grain size distribution in Halley solids as well as insight into the association of carbonaceous and non-carbonaceous materials. For convenience we will refer to the carbonaceous component as CHON and the non-carbonaceous component as silicate, although the latter may include other types of mineral. We will consider the analysed Halley particles to be aggregates of individual nanometre- to micrometre-sized 'grains', the subunits that are the actual building blocks of Halley and its dust particles. There are two likely ways the CHON and silicate components could be incorporated in grains and assembled to form particles. In the first case, the grains are either pure CHON or pure silicate. If the grains have either a narrow size range or a steep size distribution, then the C/R ratios of the aggregates would converge with increasing particle size. Figure 1 shows no narrowing of C/R with increasing ion count. This implies either that CHON and silicate grain size distributions are rather flat, to allow appreciable numbers of whole particles to be dominated by a single large CHON or silicate grain, or that grain aggregation is nonrandom, yielding highly variable ratios of CHON to silicate grains. In the second case, CHON and silicate may be intimately mixed inside individual grains, either as inclusions or as exterior coatings, as in the Greenberg cometary dust model, which postulates organic mantles on silicate grains and successfully explains many of the properties of cometary solids²⁴. A core-mantle grain structure has been tentatively inferred from the PUMA data^{4,17,25}, but it is not consistent with the wide range of observed C/R ratios if individual grains have restricted C/R fractions, as would occur with constant-thickness mantles on cores of similar size. This kind of model can be made to fit the data only if the CHON mantles or inclusions vary greatly in thickness or are depleted to varying degrees by sublimation, yielding a broad range of C/R ratios at all particle size ranges.

Most of the usable PUMA and PIA data were taken within the 20,000-km-radius zone where sublimation of C- and O-bearing material in the dust was observed^{1,5,26–29}. Data sets B and C contain too few particles for this discussion, but the set

A histogram in Fig. 2a is very asymmetric, being steeper for low-C particles and flatter for high-C particles. Figure 1 shows a near-cutoff below the 45° line where $C/R \approx 0.4$, reflected in the steep slope of Fig. 2a near this value. This line could define the break between particles that still retain various amounts of organic volatiles and those that have lost all of their volatile

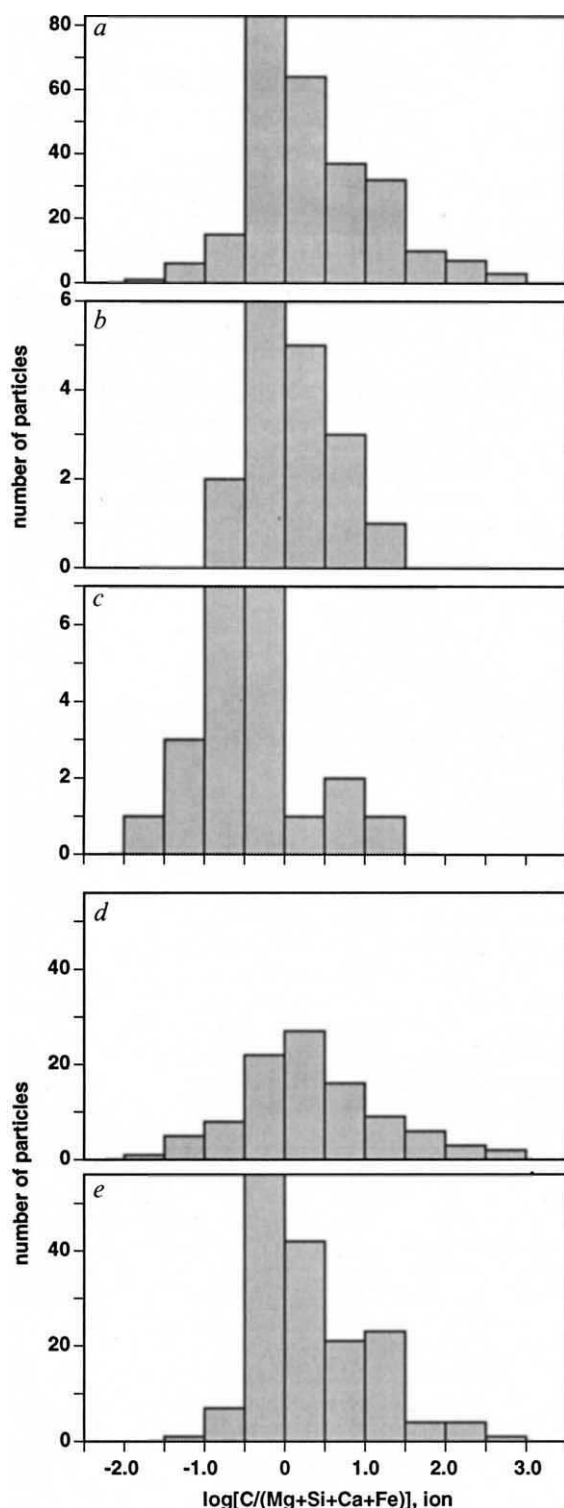


FIG. 2 Histograms of the proportion of carbon to rock-forming elements. a, Set A: PUMA-1 compressed modes, wide energy window; b, set B: PUMA-1 uncompressed mode, wide energy window; c, set C: PIA uncompressed mode; d, 'large' particles from set A—those that lie above the dotted line shown in Fig. 1 (99 particles shown); e, 'small' particles from set A—those that lie below the dotted line in Fig. 1 (159 particles shown).

CHON and retain only a residuum of refractory CHON. Particles that have lost most volatile CHON material could have settled into the relatively narrow part of the distribution near $C/R \approx 1$. Figure 2d and e shows that the smaller particles have a narrower and more asymmetric C/R distribution than the larger particles. Sublimation would be most complete in the smaller particles because of their higher surface-to-mass ratios^{30,31}, and they have a narrower final C/R distribution. PUMA-1 data therefore seem consistent in more ways than one with sublimation of volatile CHON material. □

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Seasonal distribution of African savanna fires

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SAVANNAS consist of a continuous layer of grass interspersed with scattered trees or shrubs, and cover ~10 million square kilometres of tropical Africa^{1,2}. African savanna fires, almost all resulting from human activities, may produce as much as a third of the total global emissions from biomass burning³. Little is known, however, about the frequency and location of these fires, and the area burned each year⁴. Emissions from African savanna burning are known to be transported over the mid-Atlantic, south Pacific and Indian oceans^{5,6}; but to study fully the transport of savanna fire emissions, the spatial and temporal variations in

regional savanna burning and the seasonality of the atmospheric circulation must be considered simultaneously. Here we describe the temporal and spatial distribution of savanna fires over the entire African continent, as determined from night-time satellite imagery. We find that, contrary to expectations, most fires are left to burn uncontrolled, so that there is no strong diurnal cycle in the fire frequency. The knowledge gained from this study regarding the distribution and variability of fires will aid monitoring of the climatically important trace gases emitted from burning biomass.

The night-time low-light satellite imagery used here was acquired by the Defense Meteorological Satellite Program (DMSP) Block 5 satellites during 1986 and 1987. The satellites provide imagery primarily for use in weather forecasting, in snow and ice boundary observation, and in cloud studies^{7,8}. But night-time imaging of the Earth also records man-made and natural lighting such as cities, fires, lightning and auroras⁸. We used local midnight imagery to observe light sources on the Earth's surface in the region of Africa shown in Fig. 1. The upwelling terrestrial light is measured in the 0.4–1.1 μm range at a resolution of 2.7 km. One problem with the use of DMSP imagery is that variations in intensity cannot be quantitatively interpreted, as the images are archived in photographic form, rather than in digital form, and calibration after processing is impossible. Even so, because of the high contrast between the background and the light sources, the light sources can easily be distinguished qualitatively and mapped. Cities are isolated and removed, as their location does not vary from month to month, leaving only the time-varying light sources. Throughout the study years, visible imagery was obtained for every fourth day on average. Although these images are not spread out evenly, the temporal distribution through most months is good. We produced mosaic images showing light from the time-varying sources averaged over each month. We also examined the daily spatial distribution of fires for each month to check that each

mosaic is representative of the actual burning throughout the month.

Analysis of monthly DMSP imagery during 1986 and 1987 indicates that January is the peak season for African savanna burning north of the Equator. The January precipitation analysis shows nearly all of the savannas north of the Equator receiving less than 25 mm of precipitation. The Northern Hemisphere savannas burn extensively during this time of year and have been widely studied on the regional scale^{9–11}. In the January DMSP composite images (Fig. 2a), a wide band of fires can be seen stretching across the savannas south of the Sahara desert. The 'speckled' light sources represent the locations of active fires sweeping across the continent. The northern edge of this active fire band has been reported previously⁸. The majority of these fires are initiated by human activities^{5,6}.

During the first half of the year, the dry season shifts from the Northern Hemisphere to the Southern Hemisphere. In February and March, precipitation increases gradually from south to north across the Northern Hemisphere African savannas, and there is a noticeable drop in the frequency of fire activity. By April, few lights exist across the entire African continent except in populated and industrial areas, and savanna burning in both hemispheres is at a minimum. From March to June, drier conditions spread over the Southern Hemisphere savannas from Namibia eastward towards the interior of southern Africa, and fire activity increases rapidly over the interior southern Africa savannas. By May, burning activity is widespread in Angola, Zambia, southern Zaire and Zimbabwe. In June, burning is at a peak in southern Zaire (Fig. 2b and c) with a sinusoidal pattern defining the northern edge of the fires. This pattern follows the ecosystem boundary between sub-humid forest and the equatorial tropical rainforest in north and central Zaire, and also appeared in 1979 DMSP imagery. Some fire activity is also apparent in eastern Africa in the nation of Tanzania, but little activity is identifiable in Mozambique to the south.

From July to October, savanna burning increases in the eastern countries and wanes in some western and interior nations south of the Equator. During July and August, the fire activity slowly drops off in Zaire due to increasing precipitation, there is little change in fire activity over Angola, and fires increase in Tanzania. Through the months of August and September, drier conditions extend to the eastern coast of southern Africa, and fire activity increases in Tanzania and Mozambique (Fig. 2d). In 1987 burning activity in Angola dropped off substantially by September, but in 1986 it required a few more weeks because precipitation increased more slowly in that region. In October, the greatest fire activity across the southern African savannas occurs in the southeastern nations. During October, moist conditions return to most of the interior nations south of the Equator,

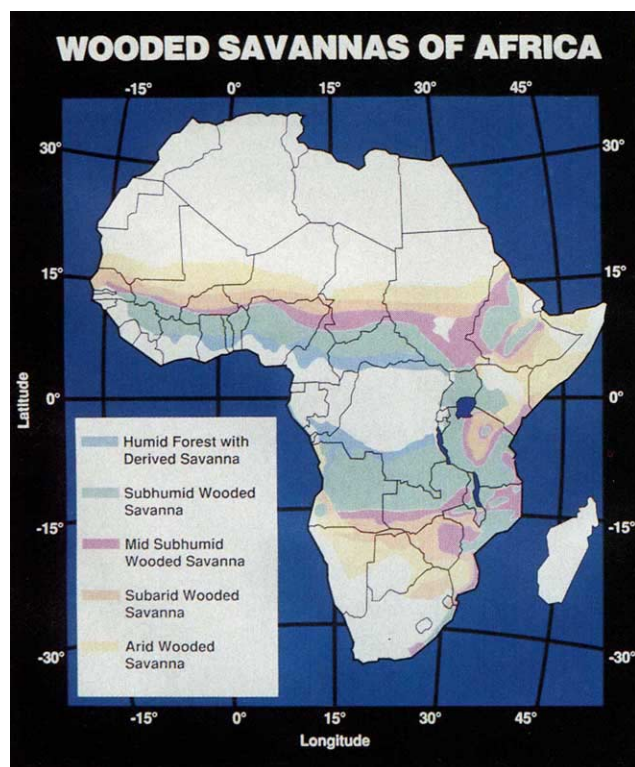


FIG. 1 A gradual transition of the savannas, influenced primarily by precipitation, takes place outward from the tropical rain forest of equatorial Africa, with wooded humid savannas giving way to savanna grasslands, semi-arid grasslands, and desert, respectively.

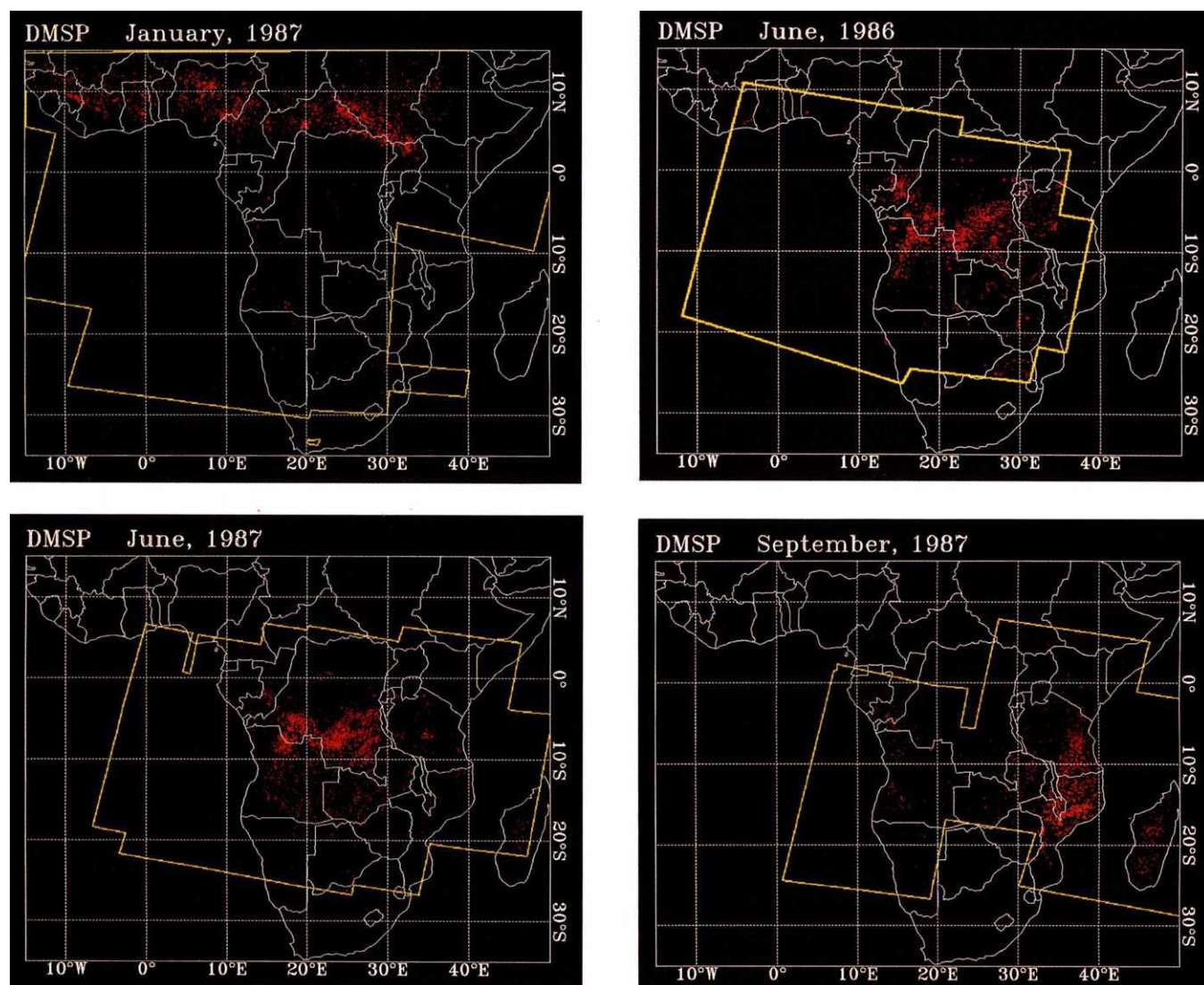


FIG. 2 Monthly DMSP mosaics for 1987. Each scene shows red 'dots' on a black background with a superimposed white outlined map of Africa. The red dots map the locations of low-level light emitted from fires. The yellow

polygon outline delineates the outer boundary of the available clear-sky satellite coverage used to produce the mosaic.

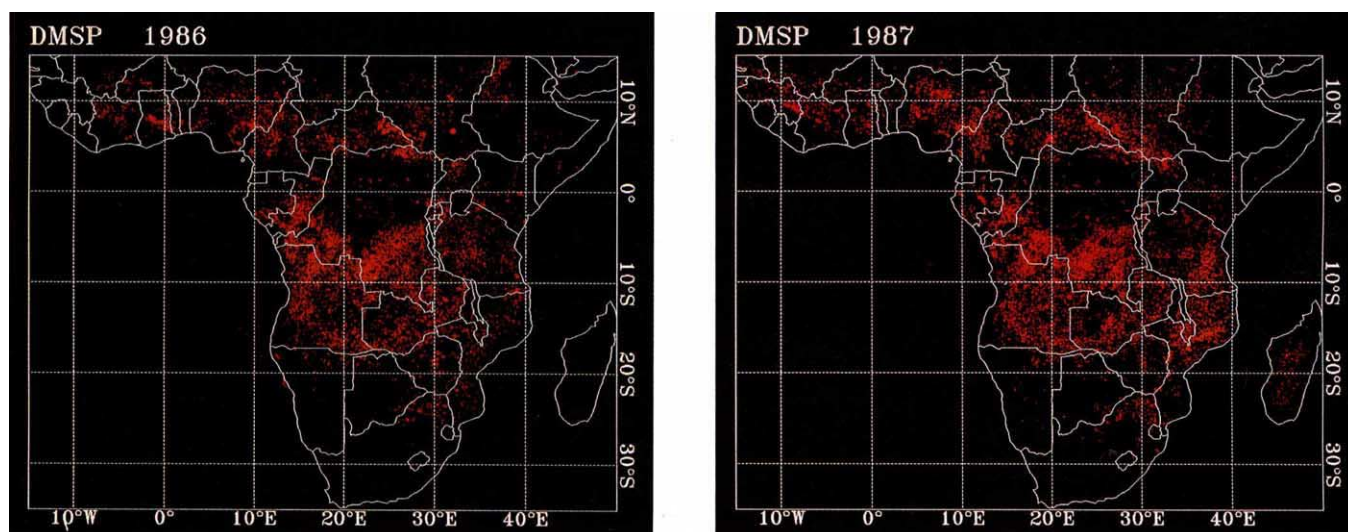


FIG. 3 1986 and 1987 DMSP mosaic, consisting of all the monthly mosaics and showing the distribution of fire activity across all of the

African savanna regions.

and dry conditions remain along a path from the coast of Namibia to Lake Malawi in the east. Fire activity seems most widespread in Tanzania, and is decreasing in neighbouring countries. This observation is consistent with results from the 1984 measurement of air pollution from satellites (MAPS) shuttle experiment which flew in early October and measured the highest middle tropospheric carbon monoxide concentrations over the southeast African nations¹². Photographs taken during the 1984 MAPS shuttle flight also showed many active fires burning in Mozambique^{3,12}.

Satellite coverage was scarce or unavailable for the remaining months in the year, so that the demise of the Southern Hemisphere African savanna burning cycle and onset of the Northern Hemisphere African savanna burning cycle could not be analysed. November 1986 imagery was available (but limited) and showed no fire activity in the west and central nations in the southern African continent. From the actual and mean precipitation patterns and the previous observations, it is expected that fire activity south of the Equator will continue along the east coast nations into November but will cease by December. At the same time, fire activity will return to the interior African savannas north of the Equator first, and spread to the far west later in December. Hence, the annual burning cycle of the African savannas continues.

All of the monthly composites for each year were combined (Fig. 3) to illustrate the extremity of savanna burning in Africa. From the annual mosaics it is immediately obvious that a large percentage of African savanna is affected annually by fire and that the overall patterns are the same. The regions that show the highest fire frequency are southern Zaire, Angola, northern Mozambique, southern Tanzania, eastern Central African Republic and southwestern Sudan. In areas such as southern Zaire (in June) where the fire frequency is extremely high, the burned areas are more likely to be homogeneous in coverage. The spatial distribution of Northern Hemisphere African fires is consistent with the work of Goldammer¹³ (based on the spatial extent of the northern savannas). From our multiyear study (including some 1979 DMSP imagery), the examination of the mean¹⁴ and actual precipitation patterns, the knowledge that the burning is the result of enhancing agricultural productivity and is tied to a seasonal cycle, and the consistency with the MAPS mission data and photography, it seems that the overall fire distribution does not vary much from year to year. This can probably be attributed to the distribution of the human population, which, in an agricultural society, is not likely to shift rapidly on a year-to-year basis. Any interannual variability is most probably attributable to variations in the precipitation patterns.

Two other observations are made from the DMSP imagery. There is a great deal of fire activity well into the night, and the night-time frequency and distribution of fires resemble those mapped during the daytime in some regional studies. Once the fires are started, in most cases they are left to burn uncontrolled, and as the satellite imagery shows, fire frequency may not be as strongly diurnal as is commonly believed. The DMSP imagery occasionally shows random fires in the rainy areas. Although random burning may take place throughout the year, the fires

are most frequent during the dry period when the herbaceous material has completely withered.

It is readily apparent that to study African savanna burning thoroughly, an extensive portion of the African continent must be examined and, for remote sensing investigations, a substantial volume of imagery must be acquired. Because of the extent of the savannas and the difficulty of working with such a volume of satellite imagery, the continental distribution of African burning has remained largely unstudied. Previous remote sensing investigations of African savanna fires have been regional in scope, not recognizing the shifting distribution of the fires such as the eastward progression of fire activity across the Southern Hemisphere savannas, or establishing its relationship with atmospheric circulation. Emission estimates from savanna burning have been based largely on statistical reports, which contain a great deal of uncertainty. Knowledge of the temporal and spatial variability of the African savanna fires, as shown here, can greatly reduce the extent of satellite coverage required to determine and monitor the total area burned throughout the year. The reduction in required satellite coverage makes it feasible to monitor and estimate the extent of the burned savanna, and, from the area estimates, the atmospheric trace gas emissions can be derived.

The transport of African savanna fire emissions depends on time, geographical location, altitude and winds. Given the expected geographical location of the fires in the Southern Hemisphere, fire emissions are more likely to be transported towards the mid-Atlantic Ocean. Emissions from savanna fires that burn in September and October around Tanzania are more likely to be transported westward towards the Atlantic Ocean rather than the Indian Ocean. Emissions from Northern Hemisphere fires are almost always transported to the west, but cross-hemispheric transport largely depends on the time of year. The wind flow also varies with altitude, and emissions at higher altitudes are more likely to be transported to the west by the trade winds. Atmospheric instability is a primary mechanism for carrying savanna fire emissions to higher altitudes, but under some atmospheric conditions, large and intense fires can inject the emissions to higher altitudes as well. Knowledge of the savanna fire location relative to the wind flow and zones of instability is extremely important in evaluating savanna fire emission export off the African continent.

Savanna burning predates the presence of humans in Africa, the result of lightning-initiated fires, but human population pressures have greatly increased its extent and frequency¹³. With the increase in fires comes the increased environmental and climatic impact of trace gas cycling and photochemistry, greenhouse gas production, acid rain deposition, and the influence of fire-produced aerosols on tropical cloud behaviour. Only through the combined knowledge of the distribution of African savanna fires, fire emission estimates and atmospheric circulation can we further our understanding of how the fire emissions affect both the biosphere and the atmosphere. As a step towards that understanding, this study demonstrates the spatial and temporal (seasonal and daily) shifts in African savanna burning and should help satellite coverage to be tailored to maximize its efficiency for estimating burned savanna. □

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Geochronological evidence supporting Antarctic deglaciation three million years ago

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THE response of the Antarctic ice sheets to increased global temperatures is an important unresolved issue in the assessment of future climate change. In particular, considerable controversy exists as to whether the East Antarctic ice sheet suffered extensive deglaciation during the mid-Pliocene epoch (~3 Myr ago), when temperatures were only slightly warmer than today. Although the ice sheet is widely assumed to have existed in something like its present form for the past 14 Myr (ref. 1), marine diatoms eroded from the Antarctic interior have been found in glacial till deposits high in the Transantarctic Mountains^{2,3}, and have been biostratigraphically dated at ~3 Myr before present. This age has been disputed⁴ because it implies marine deposition in the Antarctic interior, and hence substantial deglaciation, at a time when other evidence has been marshalled for the persistence of cold, polar conditions⁴. Here we report K–Ar and ⁴⁰Ar/³⁹Ar ages for a volcanic ash bed in diatom-bearing glaciomarine strata cored in Ferrar Fiord (East Antarctica) by the CIROS-2 drill-hole⁵, which confirm the age of the diatoms at ~3 Myr, and hence also confirm the mid-Pliocene deglaciation.

The established view of Antarctic glacial history during the Cenozoic period is that considerable cooling occurred ~40 Myr

ago, with extensive ice first covering the continent ~14 Myr ago, and persisting continuously to the present¹. This is based on oxygen isotope variations in calcareous microfossils from deep-sea cores^{6,7}, and geomorphic evidence from the Transantarctic Mountains of cold climates dating back at least 11 Myr, including several cold desert pavements^{4,8}.

Recent work, however, has described warm mid-Pliocene marine incursions in the area around McMurdo Sound^{9–11}, which includes Ferrar Fiord (Fig. 1). These incursions have been age-calibrated by potassium–argon (K–Ar) dating of basalt flows and tephra^{12,13}. But the crucial evidence for mid-Pliocene deglaciation of the Antarctic continent derives from the discovery, in glacial beds sourced from the East Antarctic interior, of microfossils (diatoms) that include taxa said to be only ~3 Myr old^{2,3}. This age was based on *Thalassiosira insigna* and *T. vulnifica*¹⁴, known to occur together only in the age range 3.1 to 2.5 Myr in a well-dated sequence cored on Deep-Sea Drilling Program leg 71 in the South Atlantic Ocean¹⁵. This age range is widely accepted by diatom workers¹⁶, and has since been confirmed from Ocean Drilling Program (ODP) cores on leg 120 in the south Indian Ocean¹⁷. The total range of *T. vulnifica* is also well dated there at 3.1 to 2.2 Myr.

These diatoms have been extracted with other microfossils, from semilithified glacial till of the Sirius Group at several locations along the Transantarctic Mountains (Fig. 1a). The till has all the features of basal glacial debris¹⁸, and the diatoms in it are therefore considered to have been eroded, transported and deposited by glacier ice. The distribution of the till, old ice flow directions from till fabric¹⁸ and flow models for an expanded ice sheet^{19,20} indicate that the source of the till is from the interior of East Antarctica (Fig. 1), a region that must have been ice-free and below sea level when the Pliocene marine diatoms, later reworked into the Sirius Group, were living. Because this would require mid-Pliocene deglaciation, which is at variance with circumstantial evidence for persistent cold in Antarctica in Pliocene times²¹, some have suggested⁴ that the diatoms are of mid-Pliocene age only in lower latitudes, and are much older in the Antarctic region.

We present here a radiometric age calibration of the Sirius

TABLE 1 Age data and grain compositions for 32–63 µm separated fractions of the CIROS-2 ash layer at 125 m b.s.f.

a, Conventional K–Ar age data								
Sample details and composition (%)*								
Fraction:	Glass	Feldspar	Quartz	Other	K (wt%)	⁴⁰ Ar (radiogenic) (nL g ^{−1})	Total	Age† (Myr)
Whole sample	72.0	8.5	12.8	6.7	—	—	—	—
Fraction A	88.9	2.1	1.9	7.0	2.226	1.249	26	14.38 ± 0.30
(mainly volcanic and glassy rock fragments)						1.292	26	14.87 ± 0.35
							Mean	14.63 ± 0.32
Fraction B	22.7	26.9	43.8	6.6	3.150	31.77	78	243 ± 3
(mainly basement quartz/feldspar)								
Fraction C	97.0	1.0	—	—	2.122	0.597	25	7.23 ± 0.20
(purified volcanic glass)							30	7.17 ± 0.20
							Mean	7.20 ± 0.20
Fraction D	98.3	0.4	1.3	—	2.163	0.283	22	3.36 ± 0.17
(very pure, 100-mg glass sample)								
b, ⁴⁰ Ar/ ³⁹ Ar age data								
Material	Analysis	Age (Myr)‡						
Anorthoclase crystal	Total fusion	2.75 ± 0.16						
Anorthoclase crystal	Total fusion	2.76 ± 0.72						
Anorthoclase crystal	Total fusion	2.81 ± 0.21						
		Mean 2.77						
Feldspar	Total fusion	6.23 ± 0.06						
Glass concentrate	Plateau	2.91 ± 0.11						
(~10,000 grains)								

* % for grain types by counting 6,000 points (fractions A and B) and 300 points (fractions C and D) on grain mounts (magnification ×10);

† Decay constants: for ⁴⁰K, $\lambda_{\beta} = 0.4962 \times 10^{-9} \text{ yr}^{-1}$; $\lambda_{\epsilon} = 0.581 \times 10^{-10} \text{ yr}^{-1}$. Isotope abundance ⁴⁰K/K = 0.01167%; errors are two standard deviations. For experimental techniques, see ref. 31.

‡ Age errors are one standard deviation. Fish Canyon Tuff sanidine (27.84 Myr) used as neutron flux monitor.

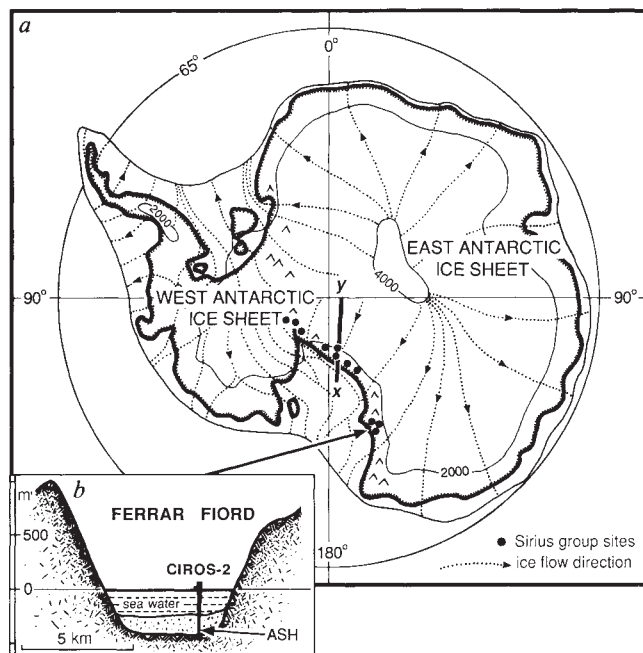


FIG. 1 a, Antarctica, showing the Transantarctic Mountains and the main areas where ice-deposited Sirius Group strata containing 3-Myr-old marine diatoms have been found. Ice thickness contours and flow lines are for the larger ice sheet required to deposit the Sirius Group debris and are taken from ref. 20. The flow pattern indicates an East Antarctic source for the diatoms, and hence marine sedimentation there ~3 Myr ago. b, Cross-section of Ferrar Fiord. The sediment fill includes both ice- and water-laid sand and mud with scattered pebbles. The core also contains a volcanic ash layer 0.3 m thick at 125 m b.s.f., and dated here by the K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ methods at ~3 Myr. The ash lies within an interval from 135 to 105 m b.s.f. dated from Southern Ocean diatom zones as ranging from 3.1 to 2.2 Myr (see Fig. 2), thus validating the use of deep-sea marine diatom biostratigraphy for Pliocene strata on the Antarctic continent. c, Cross-section from the East Antarctic interior through the Transantarctic Mountains to the Ross Sea, showing the location of Sirius group deposits and their inferred source in the interior. In drawing the section we presume that the Transantarctic Mountains were at their present elevation during Sirius deposition, but they may have been much lower at that time³⁰.

Group diatoms that shows the mid-Pliocene biostratigraphical age to be roughly correct. The marine diatom species *T. vulnifica*, whose total stratigraphic range defines the combined *T. insigna*-*T. vulnifica* zone and the *T. vulnifica* zone, is present in the CIROS-2 core from 137 to 105 m below the sea floor (m b.s.f.)¹¹. These zones form the upper part of a sequence of zones recognized and palaeontologically dated from Southern Ocean drill cores (Fig. 2). The interval containing *T. vulnifica* includes a 30-cm-thick volcanic ash layer at 125 m b.s.f. (ref. 5), which makes the dating possible. The ash layer lies within a slightly stratified diamictite (mixture of mud, sand and scattered pebbles) deposited from persistent melting of basal glacial debris just seaward of the grounding line of an ancestral Ferrar glacier²². The low proportion of nonvolcanic material in the ash (less than 30%, Table 1) indicates rapid deposition and preservation after eruption.

The ash mainly consists of fresh, dark brown or colourless silt-size shards with no chemical etching at the margins. The minor proportion is feldspar, quartz and biogenic silica (diatoms and sponge spicules). Some glass shards are vesicular and contain unzoned microphenocrysts of plagioclase, clinopyroxene, olivine and titanomagnetite. Electron microprobe analyses of glass shards (Table 2) reveal three distinct compositions, which correspond to glass colour. Using the IUGS classification scheme²³, we classify the dark brown shards as phonotephrite, light brown shards as mugearite and clear shards as benmorite. The three distinct compositions suggest that the ash layer was produced by multiple volcanic eruptions, although a single eruption of mixed magma is possible.

Initial conventional K-Ar dating on bulk (~1 g) volcanic ash

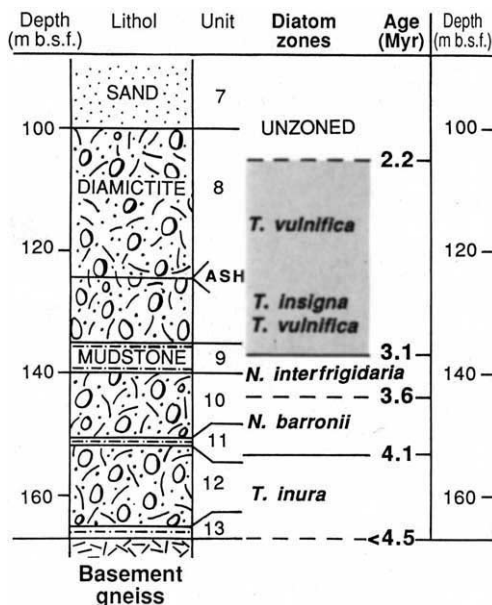


FIG. 2 Lithological column for the CIROS-2 drillhole, Ferrar Fiord, showing the diatom zones and boundary ages established by D. M. Harwood (personal communication, and ref. 14) and from ODP leg 120 data¹⁷. The range of *T. vulnifica* (shaded interval) is well dated in the Southern Ocean at between 2.2 and 3.1 Myr ago¹⁷.

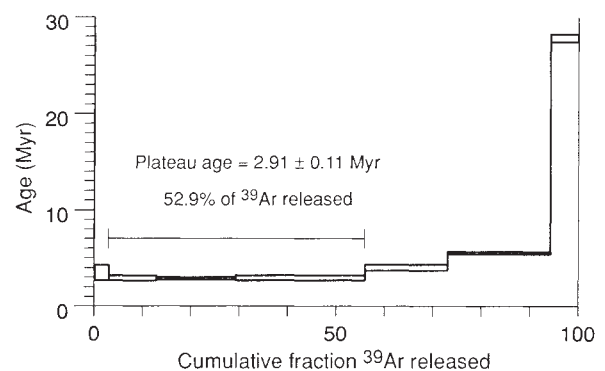


FIG. 3 Argon release spectrum for a purified glass concentrate from coarse silt-size volcanic ash from the CIROS-2 drillhole.

TABLE 2 Electron microprobe analyses of glass types

Glass type	Brown (N=15)	Light brown A (N=5)	Colourless B (N=9)
IUGS name	Phonolitic tephrite	Mugearite	Benmorite
Element	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)
SiO ₂	47.09 (0.74)	54.70 (0.86)	60.52 (0.83)
TiO	3.64 (0.34)	1.82 (0.15)	1.47 (0.05)
Al ₂ O ₃	17.06 (0.32)	19.49 (0.13)	16.91 (0.72)
FeO*	9.80 (0.84)	6.46 (0.03)	3.97 (0.16)
MnO	0.23 (0.10)	0.36 (0.06)	0.20 (0.01)
MgO	3.50 (0.23)	1.57 (0.08)	1.50 (0.12)
CaO	8.09 (0.47)	4.73 (0.63)	3.48 (0.32)
Na ₂ O	6.06 (0.37)	4.07 (0.59)	3.05 (0.71)
K ₂ O	3.06 (0.25)	2.60 (0.25)	4.65 (0.37)
Cl	0.11 (0.03)	0.22 (0.09)	0.13 (0.02)
Total	98.64	96.02	95.88
(Anhydrous)			
Water	1.36	3.98	4.12
(by subtraction)			

Samples are from the volcanic ash bed at 125 m in the CIROS-2 core. Analyst was R. H. Grapes on the JEOL superprobe at Victoria University.

* All iron as FeO.

samples gave ages of 11.9, 17.0 and 22.9 Myr, suggesting a contamination by old feldspar from basement detritus (~450–500 Myr; ref. 24) intermixed with the ash. Microscopy suggested that the ash would have only two groups of grain types contributing argon to the age equation: the young volcanic glass with its included microphenocrysts and volcanic feldspar with fringing glass, and 'basement' feldspar. We then magnetically separated a 32–63- μ m-sized sample fraction into glass-rich (contemporaneous volcanic) and feldspar-rich (basement) components (fractions A and B, Table 1a). Mineral proportions were determined by point-counting grain mounts. Using the K–Ar ages for fractions A and B, 14.6 ± 0.3 and 243 ± 3 Myr respectively, we solved simultaneous equations to obtain an age of 3.0 ± 0.4 Myr for the volcanic endmember and 445 ± 58 Myr for the basement endmember.

The high age errors, which largely reflect the counting error inherent in determining the proportion of glass to basement feldspar, motivated us to purify fraction B further, obtaining a small (~1 g) fraction C, and a very small (<0.15 g) fraction D of purified volcanic glass (Table 1). These yielded ages of 7.20 and 3.36 Myr respectively, confirming our view of contamination by old (~450 Myr) feldspar, and our estimate of 3 Myr for the glass component of the CIROS-2 ash.

At the same time $^{40}\text{Ar}/^{39}\text{Ar}$ dating techniques²⁵ were applied to four single feldspar crystals from the ash. Each crystal was laser-fused and dated individually, thus obviating contamination by older material²⁶. Three out of four crystals, identified as anorthoclase, yielded statistically indistinguishable ages, averaging 2.77 Myr (Table 1b), which we interpret as the eruptive age of the phonotephrite component of the ash. The fourth feldspar is considered detrital and from an earlier eruptive phase. In addition, a 3-mg glass sample, purified by a combination of magnetic and density liquid separation techniques, was fused to yield a $^{40}\text{Ar}/^{39}\text{Ar}$ spectrum by incremental laser heating to yield a plateau age for the glass (Table 1b). The age spectrum of the glass concentrate has a low-temperature plateau²⁷ at 2.91 ± 0.11 Myr and a higher-temperature peak of 27.8 Myr considered to represent much older contaminant material. The 2.9 Myr age is regarded as an average eruptive age for the glass.

Although volcanic glass is generally an inferior material for $^{40}\text{Ar}/^{39}\text{Ar}$ and K–Ar dating (because of its low argon retentivity, vulnerability to argon loss, and potassium mobility during dehydration and devitrification) the materials here are well characterized fresh glass with little, if any, burial heating or alteration. The concordance of $^{40}\text{Ar}/^{39}\text{Ar}$ feldspar and glass ages, and the K–Ar glass model age, and the convergence of the purified glass ages on the same value, provide in our view conclusive evidence of a mid-Pliocene (2.8 ± 0.3 Myr) age for the CIROS-2 ash. This also dates the middle of the total time range of *T. vulnifica* for the region, and a mid-Pliocene marine incursion into the Antarctic interior. This incursion was followed by temperate glaciation depositing the Sirius Group, and by the development of the present polar ice sheet.

The mid-Pliocene deglaciation of Antarctica is inconsistent with the widely accepted idea of increasing thermal isolation of Antarctica and growth of the ice cap to essentially its present form in middle Miocene times¹. The survival of *Nothofagus* through to mid-Pliocene times in the Beardmore glacier area also challenges that view²⁸. It now seems more likely that for most of the past 40 Myr the Antarctic region was characterized by waxing and waning temperate ice sheets of continental proportions²⁹. The present polar ice sheet may not have formed until the latest Pliocene, about the same time as the first northern ice sheets developed. This different climate history will require us to revise our ideas of the evolution of the flora and fauna of the region, and of the role of the ice sheet in past ocean circulation. It also suggests the need to consider the stability of the Antarctic ice sheet if global temperatures rise as the level of atmospheric CO₂ reaches or exceeds that of the Pliocene. □

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New links between the Chicxulub impact structure and the Cretaceous/Tertiary boundary

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THE 200-km-diameter Chicxulub structure¹⁻³ in northern Yucatán, Mexico has emerged as the prime candidate for the Cretaceous/Tertiary (K/T) boundary impact crater³⁻⁶. Concentric geophysical anomalies associated with enigmatic occurrences of Upper Cretaceous breccias and andesitic rocks led Penfield and Camargo¹ to suspect that this structure was a buried impact basin. More recently, the discovery of shocked quartz grains in a Chicxulub breccia³, and chemical similarities between Chicxulub rocks and K/T tektite-like glasses³⁻⁶ have been advanced as evidence that the Chicxulub structure is a K/T impact site. Here we present evidence from core samples that Chicxulub is indeed a K/T source crater, and can apparently account for all the evidence of impact distributed globally at the K/T boundary without the need for simultaneous multiple impacts or comet showers. Shocked breccia clasts found in the cores are similar to shocked lithic fragments found worldwide in the K/T boundary ejecta layer^{7,8}. The Chicxulub melt rocks that we studied contain anomalously high levels of iridium (up to 13.5 parts per 10⁹), also consistent with the iridium-enriched K/T boundary layer⁹. Our best estimate of the crystallization age of these melt rocks, as determined by ⁴⁰Ar/³⁹Ar analyses, is 65.2 ± 0.4 (1 σ) Myr, in good agreement with the mean plateau age of 64.98 ± 0.05 Myr recently reported¹⁰. Furthermore, these melt rocks acquired a remanent magnetization indicating that they cooled during an episode of reversed geomagnetic polarity. The only such episode consistent with ⁴⁰Ar/³⁹Ar constraints is chron 29R, which includes the K/T boundary.

Our analysis is based on core samples of melt rocks and breccia units from two Petróleos Mexicanos exploratory wells within the Chicxulub structure: Chicxulub 1 (C1), located near the centre of the structure, and Yucatan 6 (Y6), located ~60 km south-southwest of the centre^{2,3}. Silicate rocks recovered from Y6 show a well-sorted, apparently graded breccia sequence extending from ~1,100 m below sea level (m b.s.l.) to >1,400 m b.s.l. In the upper portions of this unit the clasts are heavily altered and are the size of medium to coarse sand, grading to pebble-sized clasts at core interval Y6-N14 (1208–1,211 m b.s.l.). This polymict breccia, and the underlying melt rocks of N17 (1,295.5–1,299 m b.s.l.), are described elsewhere^{3,6,11}. Towards the bottom of the sequence are well-crystallized coherent melt rocks and coarse-grained melt breccias.

Y6-N19 (1,377–1,379.5 m b.s.l.) breccia consists primarily of ~4–8 cm clasts of fine-grained to glassy, typically altered, melt rock in a medium- to coarse-grained melt rock matrix composed mainly of pyroxene and feldspar showing little petrographic indication of alteration. The clasts contain abundant 0.2 to ~1.0 cm fragments of silicate basement rocks showing various degrees of digestion. C1-N10 (1,393–1,394 m b.s.l.) is fine- to medium-grained coherent crystalline melt rock composed of subhedral to euhedral pyroxene, feldspar, and interstitial cryptocrystalline to microcrystalline phases including opaques. There is no petrographic indication of alteration or undigested clasts in the C1-N10 samples.

Compositional similarities between glass spherules at the Haiti K/T boundary and Chicxulub rocks have been advanced

as evidence for a genetic link³⁻⁶. We also found major element compositions essentially like those of K/T ejecta spherules for the samples we have studied, including C1-N10 and Y6-N19 which show considerably less evidence of secondary chemical alteration or disequilibrium than the samples from Y6-N17 studied previously^{3,6}. Melt compositions, however, vary from andesitic to dacitic, possibly reflecting different proportions of the various target rocks⁶.

Centimetre-sized fragments of fine- to coarse-grained silicate basement are common in Y6-N14 and Y6-N19 breccias and indicate medium- to high-grade metamorphic terrain. The predominant basement rock is pink, medium- to coarse-grained granitic gneiss containing ~10% quartz, ~80% alkali feldspar and ~10% low-calcium plagioclase. An electron microprobe survey of 40 feldspar grains in two thin sections from Y6-N14 establishes a minimum compositional range for alkali feldspar and plagioclase of $An_1Ab_{96}Or_3$ – $An_2Ab_{13}Or_{85}$ and $An_3Ab_{96}Or_1$ – $An_{16}Ab_{76}Or_8$, respectively. Accessory minerals such as biotite, pyroxene or hornblende are rare in the granitic clasts, possibly as a result of alteration. Sphene, apatite and zircon exist as trace minerals. Grain boundary relationships within these fragments reveal substantial shearing and recrystallization before impact. In addition, ~20% of the phyllosilicate clasts³ in Y6-N14 contain ≤ 1 -mm-wide irregular domains of relict glasses¹¹ that are similar in major element chemistry (Table 1) to feldspars in the granitic clasts, but microprobe analyses of these glasses consistently yield low totals (~91 wt%) suggesting that they have incurred secondary hydration. In conjunction with the granitic clasts in Y6-N19 melt clasts, these mineral glasses demonstrate that granitic gneiss is a chief constituent of Chicxulub melts. Other basement clasts include quartz-mica schist, metaquartzite and rare occurrences of mylonite. These rock types are similar, in mineralogy, structural fabric and texture, to the shocked lithic clasts distributed within the K/T boundary^{7,8}. The discovery of these target lithologies at Chicxulub obviates the need for another K/T source crater, such as the Manson structure⁷ in Iowa, to account for the shocked clasts in the boundary layer.

Evidence of shock metamorphism is abundant and unequivocal in breccia clasts: one-third of the quartz grains and most feldspar grains observed in basement clasts or as xenocrysts in melt clasts contain single or multiple sets of planar deformation features. We measured up to five sets of planar deformation

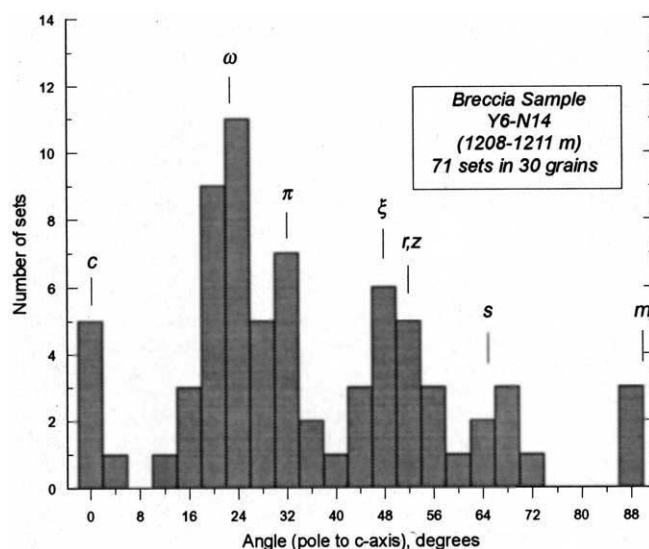


FIG. 1 Histogram showing variations in the angle between the c-axis and the pole of planar deformation features in quartz grains. Crystallographic orientations characteristic of planar deformation features in shock-deformed quartz are indicated; types A and B features are c- and ω -orientations, types C and D are r-, z-, π - and ξ -orientations.

TABLE 1 Major element analyses

	1	2	3	4	5	6	7	8	9	10
	Y6-N14 melt clast	Y6-N14 melt clast	Y6-N17 melt rock	Y6-N19-F melt matrix	Y6-N19-R melt clast	C1-N10 melt rock	Y6-N14 mineral glass	Haitian tektites	Andesite	Dacite
SiO ₂	67.9	60.9	62.3	61.2	57.6	64.4	57.9-61.7	60.3-67.9	60.0	66.8
Al ₂ O ₃	16.9	16.6	14.6	14.7	15.1	14.9	19.7-24.6	13.7-15.3	14.9	18.2
FeO	5.2	9.1	4.8	3.9	4.2	4.6	0.2-1.0	4.6-5.7	10.8	2.14
MgO	2.5	5.4	2.9	2.7	3.1	2.8	b.d.-0.8*	2.2-3.8	2.47	1.50
CaO	1.7	1.9	8.8	9.3	11.4	5.5	1.2-1.5	4.5-10.9	6.03	3.17
Na ₂ O	0.6	0.9	4.9†	2.7†	3.6†	4.3†	4.1-7.0	2.4-3.7	3.39	4.97
K ₂ O	2.7	1.6	2.5	2.9	1.8	2.7	0.1-1.0	1.0-1.8	1.18	1.92
TiO ₂	0.7	1.0	0.5	0.4	0.4	0.5	0.01-0.8	0.48-0.84	1.13	0.23
MnO	0.07	0.09	0.1	0.1	0.11	0.09	b.d.-0.05	0.0-0.18	0.20	0.06
SO ₃	0.01	0.01	0.07	0.1	0.12	0.1	b.d.-0.07			
Sum	98.3	97.5	101.5	98.0	97.4	99.8			100.1	99.0

Analyses of Chicxulub samples, Haitian tektites and typical continental rocks. Columns 1-6, electron microprobe analyses of glass beads produced by direct fusion of sample (this work). Column 7, range of electron microprobe analyses of isotropic mineral glasses (this work). Column 8, range of electron microprobe analyses of randomly selected tektites²⁹. Columns 9 and 10, typical andesite and dacite³⁰.

* b.d., below detection.

† Determined by instrumental neutron activation analysis.

features per quartz grain with a universal stage. Figure 1 illustrates that these features correspond to the distinct crystallographic orientations characteristic of shock-produced lamellae in well studied impact structures¹². The presence of types A and B features (6-10 GPa) along with types C and D (≤ 23 GPa) indicates that this breccia contains target materials derived from

several shock pressure zones, corresponding to radially varying distances from the impact point¹³. Shock mosaicism^{14,15} and diaplectic glasses¹⁵, including maskelynite, are also common in our samples.

Several small splits from C1-N10 and Y6-N19 melt rocks contain concentrations of iridium well above typical crustal concentrations. The measured Ir abundances, determined by instrumental neutron activation analysis, range from 2.5 ± 0.5 parts per 10^9 (p.p.b.) to 13.5 ± 0.9 p.p.b., consistent with the relative abundance of meteoritic material ($\sim 10^{-2} \times$ CI chondrite) in other terrestrial impact melt rocks with well characterized siderophile element signatures¹⁶. Aliquots from several grams of homogenized material were irradiated in high-purity silica tubes at the University of Missouri Research Reactor with a thermal flux of 5.5×10^{13} n cm⁻² s⁻¹ for 10 h. Counting and data reduction were done at the NASA Johnson Space Center using standard techniques¹⁷.

We analysed two different core fragments from C1-N10: one contains 6.0 ± 0.7 p.p.b. Ir, whereas two splits from the second show no detectable Ir. The spatial relationship of these two samples is unknown because this core interval consists of numerous rock fragments which have been disturbed during and following recovery. The highest Ir concentrations were measured in two splits of a single fragment from the melt rock breccia Y6-N19-R. Significant Ir enrichments also occur in three other fragments from this breccia, whereas Ir is below detection in the five remaining analyses of Y6-N19.

These abundance variations indicate that Ir is not uniformly distributed in the Chicxulub melt rocks. Y6-N19 splits were chosen to encompass the variety of melt clasts and matrix types (primarily reflecting degree of crystallization and alteration). Samples with higher Ir concentrations seem to contain more opaque, highly reflective phases than are observed in the low-Ir samples. It is possible that all our samples contain Ir at concentrations significantly above that of terrestrial crust, albeit below our detection limit (~ 1 p.p.b. Ir). Similar variations are observed at other impact structures¹⁹ and could result from nonuniform mixing of projectile in the melt sheet, fractionation during extended crystallization of the melt sheet, and/or subsequent alteration, particularly in the presence of sea water^{16,18}.

We also attempted to measure the age of the Chicxulub impact using the $^{40}\text{Ar}/^{39}\text{Ar}$ method on small single fragments of 11 fine-grained melt rock samples. Hand-picked fragments, visibly free of clasts and weighing from 0.46 to 1.83 mg each, were irradiated in the E-3 facility of the Omega West Reactor at Los Alamos National Laboratory for ~ 15 h, where they received a neutron dose of $\sim 3 \times 10^{18}$. Sanidine from the Taylor Creek

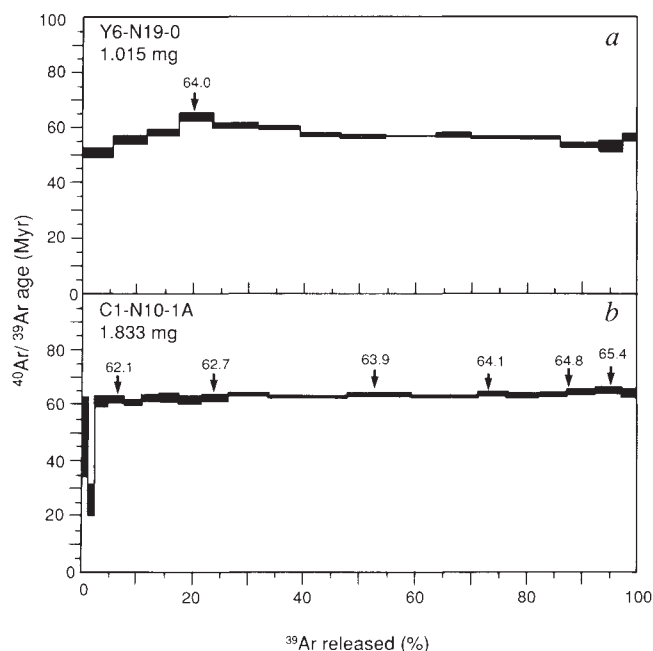


FIG. 2 $^{40}\text{Ar}/^{39}\text{Ar}$ age spectra from samples of two melt rocks from the Chicxulub impact structure. The linewidth indicates analytical errors of 1σ about the mean age of the increment. The calculated $^{40}\text{Ar}/^{39}\text{Ar}$ ages of several individual increments are shown in units of 1 Myr. *a*, The age spectrum of Y6-N19-0 has a low-temperature maximum which is typical of the age spectra of most of the altered melt rock samples. The maximum apparent age of this 'hump-shaped' segment in the ^{39}Ar release spectrum provides an estimate of the minimum crystallization age of the rock sample, 64.0 ± 1.4 Myr (1σ). Minimum age estimates acquired from release patterns of other Chicxulub samples are as follows: Y6-N14-1A, 65.4 ± 0.6 Myr; Y6-N14-1B, 62.4 ± 0.3 Myr; Y6-N17-1A, 60.4 ± 0.3 Myr; Y6-N17-1B, 65.6 ± 0.7 Myr; Y6-N17-1C, 62.8 ± 0.5 Myr; Y6-N17-2A, 60.3 ± 0.4 Myr; Y6-N19-F, 58.2 ± 0.6 Myr; and Y6-N19-J, 61.5 ± 1.5 Myr. *b*, Sample C1-N10-1A has an age spectrum with a 'near-plateau' formed by increments whose apparent ages generally increase slightly with increasing ^{39}Ar released. The last three increments have a weighted mean of 65.2 ± 0.4 Myr.

TABLE 2 Magnetic properties of Chicxulub melt rocks

Sample	Inclination (degrees)	Intensity (10^{-3} A m $^{-1}$)	Susceptibility (10^{-3} S.I. units)
Y6-N17-1A	-45.4	110.5	470
Y6-N17-1B	-40.3	83.5	350

Rhyolite (85G003, 27.92 Myr²⁰) was used as a neutron fluence monitor. After irradiation, the samples were analysed using the continuous argon-ion laser system at Menlo Park^{21,22}.

None of the samples has an age spectrum plateau indicative of a reliable crystallization age. The age spectrum for one sample, Y6-N19-R, which contains 8.3 ± 0.5 p.p.b. Ir, continually rises from the first increment (28.2 Myr) to the last (56.6 Myr). Nine of the remaining age spectra have a low- to intermediate-temperature maximum (Fig. 2a) or 'hump'²³ between about 20 and 50% ³⁹Ar released, which falls within a narrow age range. We suspect that these age spectra are the result of low-temperature alteration of the samples and that the maxima represent minimum cooling or crystallization ages.

C1-N10-1A (6.0 ± 0.7 p.p.b.), which showed the least evidence of alteration or disequilibrium in our sample suite, yielded the best estimate of the crystallization age we were able to obtain. The increments of its age spectrum are serially correlated and gradually rise from about 62 Myr in the early increments to ~65 Myr in the last few (Fig. 2b). Nonetheless, this age spectrum is very nearly a plateau over 98% of the ³⁹Ar released and the last three increments appear to level off. Consequently, we believe that these last three increments, which have a weighted mean of 65.2 ± 0.4 Myr, are probably very close to, if not equal to, the crystallization age of the sample and, therefore, to the age of the impact.

At the 95% confidence level, the 65.2 ± 0.4 (1 σ) Myr age we obtained is not different from the weighted mean of the three best ⁴⁰Ar/³⁹Ar plateau ages (64.98 ± 0.05 Myr) obtained in a simultaneous and independent study¹⁰ of ten melt rock fragments from C1-N9 and Y6-N19. This other study also obtained age spectra that resemble ours. Presumably the differences in the various age spectra obtained on different chips of the melt rocks are due to differing degrees of alteration, but the precise relation between alteration and age spectrum shape in the Chicxulub samples is unknown. Our age estimate is also statistically indistinguishable from ⁴⁰Ar/³⁹Ar ages obtained for the tektite glasses from Haiti (64.5 ± 0.1 Myr²⁴, 64.75 ± 0.08 Myr²⁵, 64.91 ± 0.06 Myr²⁶) and Mimbral, Mexico (65.07 ± 0.10 Myr¹⁰). In comparison with these results, the age of 64.0 ± 0.4 (1 σ) Myr obtained for the Haiti tektites by conventional K-Ar methods²⁷ seems to be low.

The magnetic properties of two individual melt rock fragments from Y6-N17 were analysed to evaluate whether they are consistent with a K/T age. Y6 cores were marked at the drill site to distinguish top from bottom, allowing the magnetic inclination to be determined. Stability and vectorial composition were analysed by alternating field demagnetization using a Schonstedt AF demagnetizer with maximum fields of 100 mT. The intensity and direction of natural remanent magnetization were measured with a Molspin magnetometer; susceptibility was measured with a Bartington meter.

Table 2 summarizes the magnetic properties of the two melt samples that we have analysed. Magnetization in both samples is mainly univectorial and stable; characteristic remanence resides in low-coercivity minerals of the titanomagnetite series. The mean inclination of -43° (above the horizontal) indicates that Chicxulub melt rocks crystallized and cooled during an interval of reversed geomagnetic field polarity. Recent revisions in the magnetic reversal timescale²⁸ indicate that chron 30R is probably no younger than 66.76 Myr and chron 28R no older than 64.34 Myr. Therefore, the only reversed geomagnetic period that is consistent (within 2 σ) with the crystallization ages of

Chicxulub melt rocks presented here and in ref. 10 is chron 29R, which lasted from 64.68 to 65.37 Myr and brackets the K/T boundary. □

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A new link between theropods and birds from the Cretaceous of Mongolia

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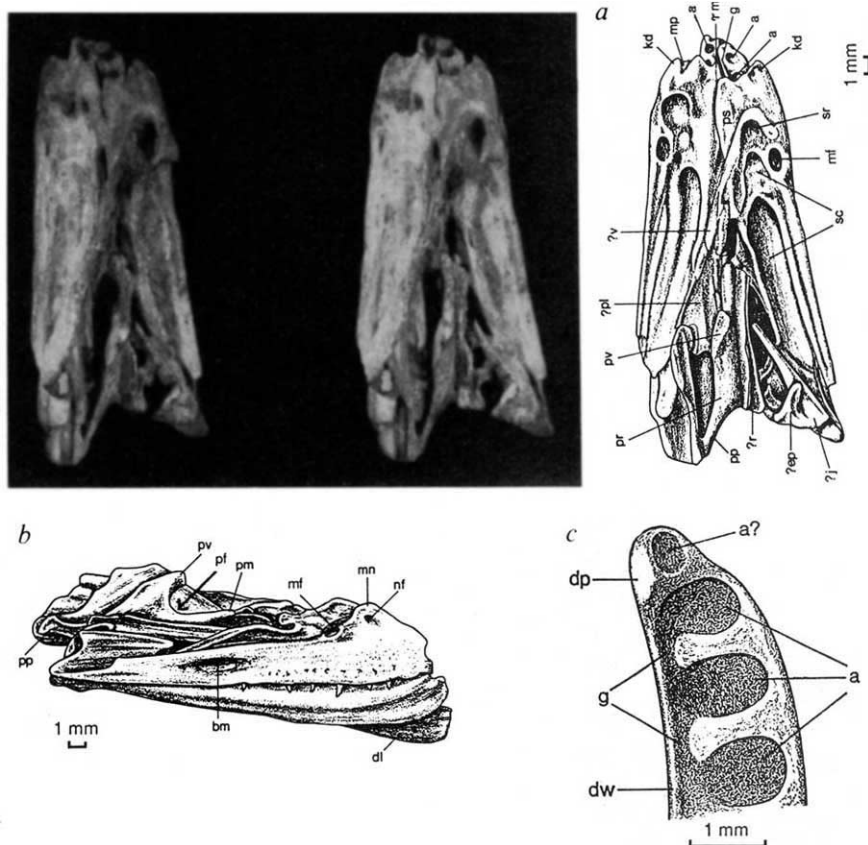
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THE origin of birds from theropod dinosaurs¹ is now broadly accepted, but there is little agreement as to which group of theropods is closest to avian ancestry². Here we report the discovery of a fragmentary skull in a collection of Late Cretaceous vertebrates from Mongolia. The skull presents a novel combination of theropod and primitive avian characters, which suggests that it belongs to the closest of the known non-avian relatives of *Archaeopteryx* and other birds. The new fossil shows consistent similarities to the troodontid theropods, for whom close avian relationships have been proposed³, and to *Baryonyx*⁴ and *Spinosaurus*⁵, two unusual theropods from the Lower Cretaceous of England and the lowermost Upper Cretaceous of Africa, respectively.

The skull was found in 1965 by the Polish-Mongolian Palaeontological Expedition in the locality that yielded an assemblage of Late Cretaceous mammals and reptiles⁶. The skull is from a juvenile individual, as revealed by the unfinished surface of the bones and the presence of barely erupted back

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FIG. 1 ZPAL MgD-II/29, *Archaeornithoides deinosaursiscus*, new species, the holotype skull in dorsal (a) and right dorsolateral (b) views, and the rostral end of the right dentary in dorsal view (c). The specimen consists of the maxillae, dentaries, and the palatal bones that had been crushed between the opposite jaws, the right palatine being the only well-preserved palatal bone. The premaxilla had fallen off before burial, because the rostral articular surfaces of both maxillae are essentially intact. The jaws had probably been bitten off from the braincase, as indicated by the presence of bite marks situated roughly opposite each other on the left dentary and the jugal ramus of the right maxilla. This accounts for the bones at the caudal end being cut across and haphazardly mixed, which makes the positive identification of most of them impossible. The bite marks point to a predator or scavenger in the size range of deltatheridiid mammals, which abound in the Bayn Dzak assemblage⁶. The total length of the skull is estimated as 5 cm. Abbreviations: a, dentary alveoli; bm, bite marks; dl, left dentary; dp, dorsal projection of the dentary; dw, medial (lingual) wall of the dentary; ep, ectopterygoid; g, paradental groove; j, jugal; kd, dorsal knob of the maxilla; mf, dorsal opening to the supraalveolar canal; mn, nasal process of the maxilla; mp, premaxillary fossa in the maxilla; nf, nutrient foramen; pf, embayment for the subsidiary palatal fenestra; pl, left palatine; pm, maxillary processes of the palatine; pp, pterygoid processes of the palatine; pr, right palatine; ps, palatal shelf of the maxilla; pv, vomeral processes of the palatine; r, parasphenoid rostrum; rm, rostromedial process of the maxilla; sc, caudal sinus of the maxilla; sr, rostral sinus of the maxilla; v, vomer. Scale bars, 1 mm.



teeth. However, it is extremely unlikely to derive from a juvenile of any known theropod owing to the unique combination of features of the maxilla and dental alveoli.

Order Saurischia
Suborder Theropoda
Archaeornithoididae fam. nov.
Archaeornithoides deinosaursiscus gen. et sp. nov.

Etymology. *Archaeornis*, a junior synonym of *Archaeopteryx*, *oides* (Greek) resembling, like, *deinosaursiscus*, a little dinosaur.
Holotype. ZPAL (Zaklad Paleobiologii) MgD-II/29 (Fig. 1).
Horizon and locality. Djadokhta Formation (Late Cretaceous: late Santonian or early Campanian⁶); Bayn Dzak (formerly Shabarakh Usu), District Dalandzadgad, Mongolia.

Diagnosis. Maxilla with a major pit on the lateral surface of the nasal process, the rostromedial process recurved ventrally, a large dorsal opening to the supraalveolar canal behind the base of the nasal process, and a broad palatal shelf enclosing a small rostral and a large caudal sinus. Dentary with interdental septa descending labio-lingually and separated from the lingual wall by the paradental groove.

A distinct theropod character of *Archaeornithoides* is the tetradactyl palatine with a medial embayment for the subsidiary palatal fenestra (Fig. 1b), which is present in all dromaeosaurid⁷ and some tyrannosaurid⁸ theropods. The premaxilla-maxilla articulation in *Archaeornithoides* resembles that in *Baryonyx*⁴, and differs from that in the carnosaurs⁹, in having two maximally knobs that line up above one another (Fig. 2). The steep ventral

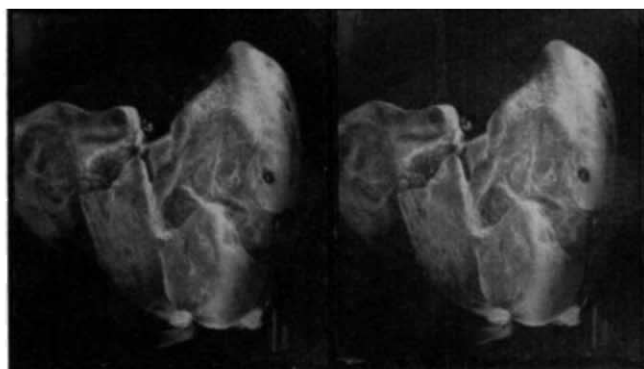
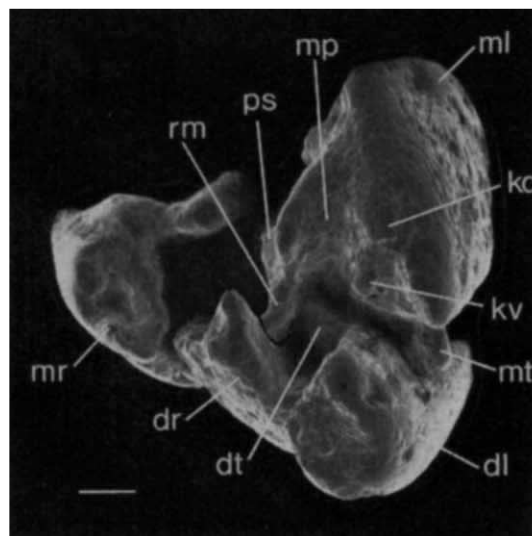


FIG. 2 ZPAL MgD-II/29, *Archaeornithoides deinosaursiscus*, new species, the holotype skull in rostral view. Abbreviations: dl, left dentary; dr, right dentary; dt, third dentary tooth; kd, dorsal knob of the maxilla; kv, ventral knob of the maxilla; ml, left maxilla; mp, premaxillary fossa in the maxilla; mr, right maxilla; mt, first maxillary tooth; ps, palatal shelf of the maxilla; rm, rostromedial process of the maxilla. Scale bar, 1 mm.

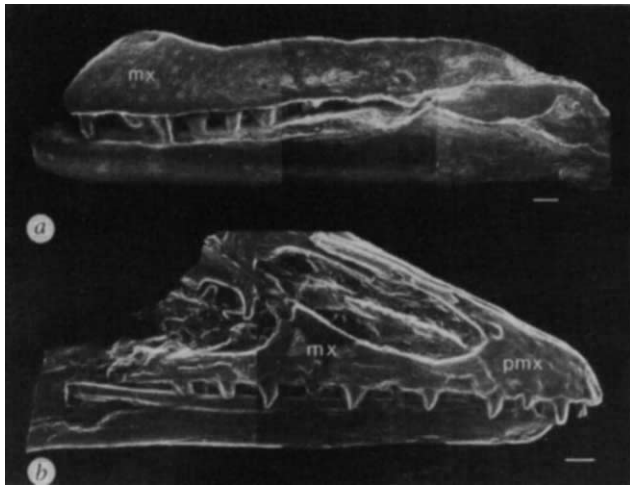


FIG. 3 The upper dentition in ZPAL Mg-II/29, *Archaeornithoides deinosaureus*, new species (a) and the Eichstätt specimen of *Archaeopteryx lithographica* (b). The count of 8–11 maxillary teeth in *Archaeornithoides*, including the barely erupted back teeth (not visible in lateral view), may have little diagnostic value as the specimen is a juvenile. But the overall form of teeth is unlikely to have been substantially different in the adult animal inasmuch as the juvenile theropod teeth are known to be scaled down versions of teeth in grown-up individuals¹¹. Abbreviations: mx, maxilla; pmx, premaxilla. Scale bars, 1 mm.

sloping of the palatal shelves in their rostral sections (Fig. 2) indicates the presence of a median ridge. Because the maxillary palatal shelves articulate rostrally with those of the premaxilla, the median ridge must have continued onto the premaxilla. The only dinosaur known to possess a median ridge in the rostral part of the palate is *Baryonyx*^{4,10}.

Archaeornithoides differs from most theropods¹¹ in lacking the interdental plates (secondary ossifications that enclose dental alveoli on the lingual side), which are known to be absent only in *Baryonyx*, *Spinosaurus*, Troodontidae and, possibly because of a secondary fusion³, in the Dromaeosauridae⁷. A striking feature shared by *Archaeornithoides* with *Baryonyx*⁴, *Spinosaurus*⁵ and Troodontidae³ is the presence of the paradental groove (Fig. 1c) that separates the interdental septa (or their remnants in the troodontids) from the lingual wall of the dentary. The dentary of *Archaeornithoides* is strikingly similar to that of *Spinosaurus*⁵ in the shape of the rostral alveoli (Fig. 1c), as well as in the irregular arrangement of the widely spaced caudal teeth.

Archaeornithoides resembles *Archaeopteryx* in having widely spaced, pointed, maxillary teeth (Fig. 3), which, in contrast to those of most theropods, are conical or only slightly compressed labio-lingually, and lack both serrations and carinae. Whereas the premaxillary and mesial dentary teeth tend to be rounded in some theropods¹¹, the maxillary teeth are known to be rounded only in very few of them, including *Baryonyx* and *Spinosaurus*. Serrations of the maxillary teeth are minuscule in *Baryonyx* and absent altogether in *Spinosaurus* and *Lisboasaurus estesi*, a species based on a single maxilla from the Upper Jurassic of Europe and recently redescribed as a maniraptoran theropod¹². All three genera have distinct carinae, however, as do other theropods. The lack of dental carinae may, therefore, be a synapomorphy uniting *Archaeornithoides* and birds.

What best distinguishes *Archaeornithoides* from any known theropod is the broad palatal shelf with its pneumatic sinuses (Fig. 1a). The shelf in *Archaeornithoides* is so expanded that the subfenestral part of the maxilla is distinctly broader than it is high, which is in marked contrast to the theropods^{9,13}, with a partial exception of *Lisboasaurus*. Among birds, a comparably expanded shelf is found in *Archaeopteryx*^{14,15} and the primitive neornithines, including *Hesperornis*^{15,16} and the palaeognaths. The expanded palatal shelf seems, therefore, to be primitive for

birds. The configuration of the small rostral sinus, the large caudal, medially bulging sinus, and the nasal process with an opening to the supraalveolar canal just behind its base, is strictly comparable in *Archaeornithoides* and *Hesperornis*¹⁵, but unknown in the theropods.

The avian features of the maxillary palatal shelf and dentition distinguish *Archaeornithoides* from all other potential sister-groups of birds², suggesting that *Archaeornithoides* is the closest known theropod relative of birds. The ramifications of this corollary for the origin of birds hinge on the phylogenetic status of the presence of a paradental groove, the lack of interdental plates and other similarities of *Archaeornithoides* to *Baryonyx*, *Spinosaurus* and troodontids. If the ceratosaurs, which show the opposite character states¹⁷, are correctly identified as the pleisiomorphic sister taxon of all other theropods^{18,19}, then the similarities of *Archaeornithoides*, *Baryonyx*, *Spinosaurus* and troodontids are synapomorphic—indicating that these taxa, and possibly *Lisboasaurus*, form a monophyletic taxon. But although 4–5 out of 6 available *Baryonyx* characters support its branching off after the ceratosaurs⁴, the *Baryonyx*–*Spinosaurus* clade²⁰ does not fit Gauthier's¹⁸ subdivision of the remaining theropods into the carnosaurs and coelurosaurs, with birds and other maniraptorans as the most recent branches of the coelurosaurs. This opens up the possibility that the avian lineage branched off further back in the theropod phylogeny than suggested by Gauthier's¹⁸ phylogenetic scheme. □

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Social contracts in wasp societies

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THE stability of social groups requires that conflicts among group members somehow be resolved. Recent models predict that subordinates may be allowed limited reproduction by dominant colony-mates as an inducement to stay and aid dominants^{1–3}. For such 'social contracts' to be evolutionarily stable, attempted reproductive cheating by dominants must be punishable³. In the eusocial paper wasp, *Polistes fuscatus*, subordinate queens that co-found nests with dominant queens usually disappear after the first workers emerge, so subordinates lay most of their reproductive-destined

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eggs just before worker emergence. Thus subordinates should be very sensitive to reproductive cheating during the latter period but relatively insensitive when worker-destined eggs are laid. Here we find in a series of egg-removal experiments designed to mimic egg-eating that subordinates do not change their aggressiveness when worker-destined eggs are removed, but that they greatly increase their aggression when reproductive-destined eggs are removed, especially when the queens are of similar size.

In primitively eusocial wasps of the genus *Polistes*, several related queens commonly cooperate in founding spring nests after overwintering^{2,4,5}. A linear dominance hierarchy develops among these females, and the most dominant female produces the majority (but not all) of the reproductive offspring (males and future queens, which mate in the autumn before the future queens overwinter)^{2,4-6}. The dominant queen regulates the reproduction of subordinates in part by eating some (on average, about 1/3) of the eggs of the latter^{4,5}.

We simulated such egg-eating by removing eggs from foundress colonies of *P. fuscatus* and examined the resulting behavioural responses. In *P. fuscatus*, co-founding queens are close relatives^{2,4,5}, with a mean genetic relatedness of 0.42–0.65 (ref. 6). Subordinates have been observed to lay an average of 33% of reproductive-destined eggs after egg eating has been accounted for⁵; this value is consistent with our observations of undisturbed colonies (4 of 12 layings of reproductive-destined eggs) as well as with that inferred from genetic data for a closely related species, *P. metricus* (13–22%; ref. 7). These mixed maternities of brood can be explained by models of the optimal degree of reproductive domination (sometimes called 'skew') by the dominant (alpha) queen. In particular, the alpha queen is predicted to forfeit direct reproduction to the beta (subordinate) queen when such an allowance provides an incentive for the beta to stay and cooperate peacefully rather than to leave and nest solitarily or fight for complete reproductive control^{2,3}.

Implicit in the optimal-skew models is that some mechanism exists whereby a queen is prevented from 'cheating' (for example, by eating all the eggs of the other queen and replacing them with her own). One possibility is that each queen monitors her own reproduction and aggressively retaliates if the other attempts to alter the skew in her own favour. According to a recent extension of the models of optimal skew³, beta queens that are more closely matched in fighting ability to their alpha queens should be allowed a greater fraction of the total reproduction and should therefore be more responsive to loss of a given number of randomly chosen eggs from the colony. Current evidence strongly suggests that body size and fighting ability are correlated in *Polistes* wasps². We predicted that subordinates of greater relative size should be more likely to retaliate when their production of reproductive brood is jeopardized, because larger subordinates should have a greater incentive for retaliating and be more effective in retaliating.

We tested these predictions by observing whether experimental removal of reproductive-destined eggs enhanced aggression between alpha and beta queens in small foundress associations of *P. fuscatus* nesting near Bedford, Massachusetts. Although the maternal origins of removed eggs were unknown, we predicted that egg removal would increase aggression on the nest between the queens because of perceived oophagy by co-foundresses. We also tested the beta queen's response to removal of worker-destined eggs, which are produced earlier in the nest-founding period^{4,5}. As these eggs produce mostly non-reproductive individuals, we predicted that the beta queen would exhibit less intense retaliation after their removal.

Alpha and beta queens were identified informally before observation by their dominance-subordinance behaviours. Dominant queens are easily recognized by their ritual mounting of subordinates, abdominal wagging, high proportion of time on the nest, relatively frequent egg-laying behaviour, and monopolization of incoming prey^{2,4,8}. Dominance hierarchies were confirmed during control observations. Dominance never

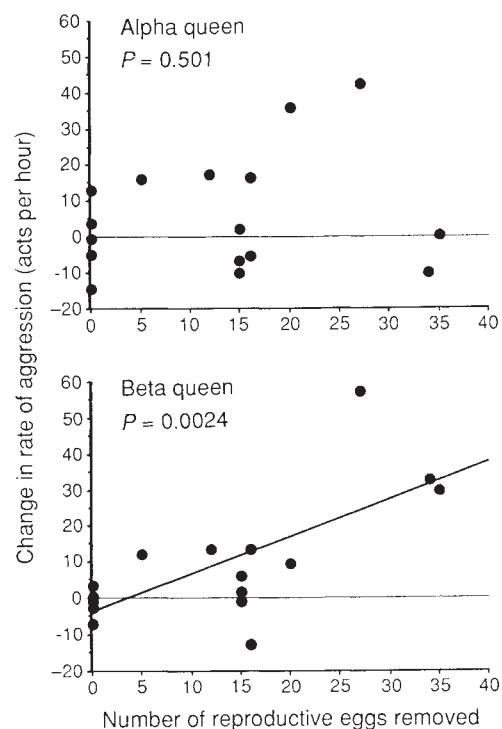


FIG. 1 Change in the rate of overall aggression between control and treatment observations, versus number of reproductive-destined eggs removed: alpha queens (top); beta queens (bottom). We observed 16 colonies (mean comb size, 59 cells), each containing 2–4 ($\bar{X}$ = 2.4) foundress queens, between 10–27 June, 1991 and between 16–18 July, 1992. All colonies were observed 0.14–2 weeks before the emergence of workers, when the first batch of reproductive-destined eggs is laid^{5,11}. We recorded all interactions among queens between 10:00–11:30 and 15:00–16:00 for two successive days. Interactions included nutrient exchange, antennation, aggressive threats (darting, chasing), and aggressive contacts (mounting with forced crouching, lunging, grappling and biting)^{4,5,12–17}. Before second-day observations (at 08:30), the comb and all queens were collected. The queens were cooled in a 4 °C refrigerator while half, all or none of the eggs were removed with forceps. (Control combs were touched with forceps.) The comb was then glued to its initial location, and the beta queen replaced. The alpha queen and other subordinates were subsequently released from their holding containers. Observations resumed after the alpha queen returned to the nest (within 1–2 h of release). Observations were blind with respect to treatment (the deeply recessed eggs were not visible from several feet away). Observations were audiotaped (eleven colonies) or videotaped (five colonies). After observations, queen body sizes were measured by wing length (tegulum base to wing tip)^{4,11}. Rate of aggression is the number of aggressive threats and contacts directed by one queen towards the other during an hour when both were on the nest^{4,5,12–17}. Changes in aggression rate were regressed on the number of eggs removed. Order of treatments was randomized. Regressions of rates of aggressive contacts also were not significant for alpha queens (P = 0.90) and were significantly positive for beta queens (P = 0.0006).

reversed after manipulation (for example, dominants continued to mount subordinates, and subordinates never mounted dominants).

We measured the level of aggression by the rate of all aggressive acts and by the rate of aggressive contacts (Fig. 1). For alpha queens, neither rate significantly increased with the number of reproductive-destined eggs removed (Fig. 1) nor with the per cent of eggs removed (P > 0.40 for regression coefficients). But beta's aggression rates markedly and significantly increased with the number of reproductive-destined eggs removed (Fig. 1; Table 1). The difference between the beta's and alpha's changes in aggression also significantly increased with the number of reproductive eggs removed (P < 0.05 for regression coefficient), further demonstrating the differential increase in beta versus alpha's aggression as more reproductive eggs were removed. Beta's rate

TABLE 1 Changes in beta queen's aggression when reproductive-destined eggs or worker-destined eggs are removed

	Change in aggression rate	P value
Removal of reproductive-destined eggs*		
Control (n=5)	-1.2 (1.7)	0.03
Treatment (n=7)	+8.1 (2.2)	
Removal of worker-destined eggs†		
Control (n=5)	+0.18 (3.6)	0.60
Treatment (n=5)	-0.92 (0.9)	

* In the comparison of changes in beta's aggression rate (acts per hour; standard errors in parentheses) between removal of 0% (control) and 50% (treatment) of the reproductive-destined eggs, data are taken from Fig. 1. Beta queens increased aggression rate significantly more when half of the eggs were removed than when none of the eggs were removed, demonstrating that beta queens could discriminate between two conditions. (The mean percentage changes in aggression rates were -12.4% for controls and +161% for treatments.) Test is a Mann-Whitney *U* test (as below).

† In the experiment comparing changes in beta's aggression rate between removal of 0% (control) and 100% (treatment) of worker-destined eggs, the numbers of eggs removed were comparable to the experiment with reproductive-destined eggs (a mean of 15.4 worker-destined eggs and 14.0 reproductive-destined eggs were removed, respectively). We observed 10 colonies ($\bar{x}$ comb size=40 cells, $\bar{x}$ =2.5 foundresses) from 10-18 June 1992. Because of a prolonged (>2 weeks) period of relatively low spring temperatures, nests at this time were much less developed than nests at the same time in 1991, as indicated by the relatively small comb sizes and absence of pupae. Thus these nests were observed earlier than 3-4 weeks before worker emergence, when predominantly worker-destined eggs were still being laid^{5,11}. Beta queens did not significantly increase their aggression when worker-destined eggs were removed.

of aggressive acts also increased with the per cent of reproductive-destined eggs removed ($P=0.012$ for regression coefficient), but beta queens did not significantly change their aggression rates when only worker-destined eggs were removed (Table 1).

The magnitude of the increase of the beta queen's aggression when reproductive-destined eggs were removed depended on its size relative to that of the alpha queen; the partial Spearman correlation between beta's change in overall aggression rate (in colonies from which eggs were removed) and the size difference between alpha and beta (number of removed reproductive eggs controlled) was significantly negative (partial $r=-0.67$; $P<0.05$). The partial correlation between beta's aggression rate and the number of reproductive eggs removed (size difference controlled) was significantly positive ($r=+0.71$; $P<0.02$). Thus beta queens (but not alpha queens) responded with greatly enhanced aggression when reproductive eggs were removed, and the magnitude of this increase was greater when the beta queen was closer in size to the alpha queen.

We also found a temporal link between aggressive acts and egg laying. The mean observed interval between the initiation of egg laying (which is preceded by a period of cell preparation) and the previous aggressive act towards the egg-layer (17 s) was significantly less than the mean interval expected if aggressive acts and egg layings were temporally independent (186 s; $P=0.043$ for five colonies in which egg layings were recorded, Wilcoxon one sample test). In summary, our results strongly support the hypothesis that beta queens retaliate against reproductive cheating by alpha queens (when such cheating threatens loss of future reproductives), especially when the beta queens are relatively large and therefore probably possess relatively high fighting ability.

An alternative hypothesis is that increased co-foundress aggression reflects only a heightened general activity of the foundresses caused by the perception that a foreign conspecific has removed eggs from the nest. Two results rule out this hypothesis. First, the hypothesis does not explain why beta, but not alpha, increases her aggression in response to egg removal, especially as recent evidence demonstrates that the alpha queen most frequently and intensely defends the colony against conspecific intruders⁹. Second, the hypothesis fails to explain why the difference in size between beta and alpha would modulate the level of aggression displayed by beta.

The strongly asymmetric response of alpha and beta to removal of reproductive eggs indicates that the two queens are not simply competing to fill newly empty cells with their own eggs. (Removal of pupae, which also induces egg-laying in the opened cells, does not significantly increase beta's level of aggression; H.K.R. and P.N., manuscript in preparation.) This asymmetric aggression is intriguing in light of the fact that beta tends to disappear from the colony in the days following worker emergence, often weeks before the end of the colony cycle^{5,10}. The alpha queen has relatively little to gain, and perhaps much to lose, by risking aggressive ejection or killing of the beta before the worker force emerges (at which time colony survivorship increases dramatically²), especially as the beta helps to prevent both nest usurpation and colony failure due to loss of all foundresses². In contrast, whatever direct reproduction is to be achieved by the beta queen must take place during her relatively short tenure on the nest. Thus beta has more incentive to retaliate against reproductive cheating than does alpha, who can reproduce in the lengthy period following beta's disappearance. (Workers contribute little if any effort to the production of reproductives in queenright colonies⁶.) In any event, our results support the hypothesis that social wasps monitor their relative reproduction, and that a fragile social contract over the allocation of direct reproduction is stably maintained by the possibility of aggressive retaliation against reproductive cheating. □

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Evolutionary games and spatial chaos

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MUCH attention has been given to the Prisoners' Dilemma as a metaphor for the problems surrounding the evolution of cooperative behaviour¹⁻⁶. This work has dealt with the relative merits of various strategies (such as tit-for-tat) when players who recognize each other meet repeatedly, and more recently with ensembles of strategies and with the effects of occasional errors. Here we neglect all strategical niceties or memories of past encounters, considering only two simple kinds of players: those who always cooperate and those who always defect. We explore the consequences of placing these players in a two-dimensional spatial array: in each round, every individual 'plays the game' with the immediate neighbours; after this, each site is occupied either by its original owner or by one of the neighbours, depending on who scores the highest total in that round; and so to the next round of the game. This simple, and purely deterministic, spatial version of the Prisoners' Dilemma, with no memories among players and no strategical elaboration, can generate chaotically changing spatial patterns, in which cooperators and defectors both persist indefinitely (in fluctuating proportions about predictable long-term averages). If the starting configurations are sufficiently symmetrical, these ever-changing sequences of spatial patterns—dynamic fractals—can be extraordinarily beautiful, and have interesting mathematical properties. There are potential implications for the dynamics of a wide variety of spatially extended systems in physics and biology.

Although it has a long history, the paradox of the Prisoners' Dilemma has recently been much studied for the light it may shed on the evolution of altruistic or cooperative behaviour^{1,2}. In its standard form, the Prisoners' Dilemma is a game played by two players, each of whom may choose either to cooperate, C, or defect, D, in any one encounter. If both players choose C, both get a pay-off of magnitude R ; if one defects while the other cooperates, D gets the game's biggest pay-off, T , while C gets S ; if both defect, both get P . With $T > R > P > S$, the paradox is evident. In any one round, the strategy D is unbeatable (being better than C whether the opponent chooses C or D). But by playing D in a sequence of encounters, both players end up scoring less than they would by cooperating (because $R > P$). Following Axelrod and Hamilton's pioneering work¹, many authors have sought to understand which strategies do best when the game is played many times between players who remember past encounters. These theoretical analyses, computer tournaments, and laboratory experiments continue, with the answers depending on the extent to which future pay-offs are discounted, on the ensemble of strategies present in the group of players, on the degree to which strategies are deterministic or error-prone (for example, imperfect memories of opponents or of past events), and so on²⁻⁶.

In this paper, we consider only two kinds of players: those who always cooperate, C, and those who always defect, D. No explicit attention is given to past or likely future encounters, so no memory is required and no complicated strategies arise. Interesting results emerge when we place these 'players'—who may be individuals or organized groups—on a two-dimensional, $n \times n$ square lattice of 'patches': each lattice-site is thus occupied either by a C or a D. In each round of our game (or at each time step, or each generation), each patch-owner plays the game with its immediate neighbours. The score for each player is the sum of the pay-offs in these encounters with neighbours. At the start of the next generation, each lattice-site is occupied by the player with the highest score among the previous owner and the immediate neighbours. The rules of this simple game among

n^2 players on an $n \times n$ lattice are thus completely deterministic. (In ref. 2 spatial arrays were briefly explored, but with a focus on the interplay among tit-for-tat and other explicitly memory-laden strategies in iterated encounters; the interest was in spatial generalizations of earlier results, such as "if a [strategy] is collectively stable, it is territorially stable".)

Specifically (but preserving the essentials of the Prisoners' Dilemma), we chose the pay-offs of the Dilemma's matrix to have the values $R = 1$, $T = b(b > 1)$, $S = P = 0$. That is, mutual cooperators each score 1, mutual defectors 0, and D scores b (which exceeds unity) against C (who scores 0 in such an encounter). The parameter b , which characterizes the advantage of defectors against cooperators, is thus the only parameter in our model; none of our findings are qualitatively altered if we instead set $P = \epsilon$, with ϵ positive but significantly below unity (so that $T > R > P > S$ is strictly satisfied). In the illustrations below, we assume the boundaries of the $n \times n$ matrix are fixed, so that players at the boundaries simply have fewer neighbours; the qualitative character of our results is unchanged if we instead choose periodic boundary conditions (so that the lattice is really a torus). The illustrations are for the case when the game is played with the eight neighbouring sites (the cells corresponding to the chess king's move), and with one's own site (which is reasonable if the players are thought of as organized groups occupying territory). As amplified below, the essential conclusions remain true if players interact only with the four orthogonal neighbours in square lattices, or with six neighbours in hexagonal lattices; the results also hold whether or not self-interactions are included.

Using an efficient computer program in which each lattice-site is represented as a pixel of the screen, we have explored the asymptotic behaviour of this system for various values of b , and with various initial proportions of C and D arranged randomly or regularly on an $n \times n$ lattice ($n = 20$ and more). The dynamical behaviour of the system depends on the parameter b ; the discrete nature of the possible pay-off totals means that there will be a series of discrete transition-values of b that lead from one dynamical regime to another. These transition-values and the corresponding patterns are described in detail elsewhere⁷. The essentials, however, can be summarized in broad terms. If $b > 1.8$, a 2×2 or larger cluster of D will continue to grow at the corners (although not necessarily along the edges, for large squares); for $b < 1.8$, big D clusters shrink. Conversely, if $b < 2$, a 2×2 or larger cluster of C will continue to grow; for $b > 2$, C clusters do not grow. The most interesting regime is therefore $2 > b > 1.8$, where C clusters can grow in regions of D and also D clusters can grow in regions of C. As intuition might suggest, in this interesting regime we find chaotically varying spatial arrays, in which C and D both persist in shifting patterns. Although the detailed patterns change from generation to generation—as both C clusters and D clusters expand, collide, and fragment—the asymptotic overall fraction of sites occupied by C, f_C , fluctuates around 0.318 for almost all starting proportions and configurations.

Figure 1 illustrates typical asymptotic patterns for two different regimes of b values. The colour coding is as follows: blue represents a C site that was C in the preceding generation; red, a D site following a D; yellow, D following a C; and green, C following a D. Thus a purely red and blue pattern would necessarily be static. The amount of yellow and green in a picture indicates how many sites are changing from one generation to the next. Figure 1a, for $1.75 < b < 1.8$, is typical of the irregular and relatively static network of 'D lines' against a background of C which arises for these b values; one gets less connected fragments of D lines or 'D blinkers' for lower b values. Figure 1b is for the interesting regime $2 > b > 1.8$, and shows the typical patterns of dynamic chaos found for almost all starting conditions in this regime. Figure 2a adds a temporal dimension to Fig. 1b, showing the proportion of sites occupied by C in successive time-steps (starting with 10% D). The asymptotic

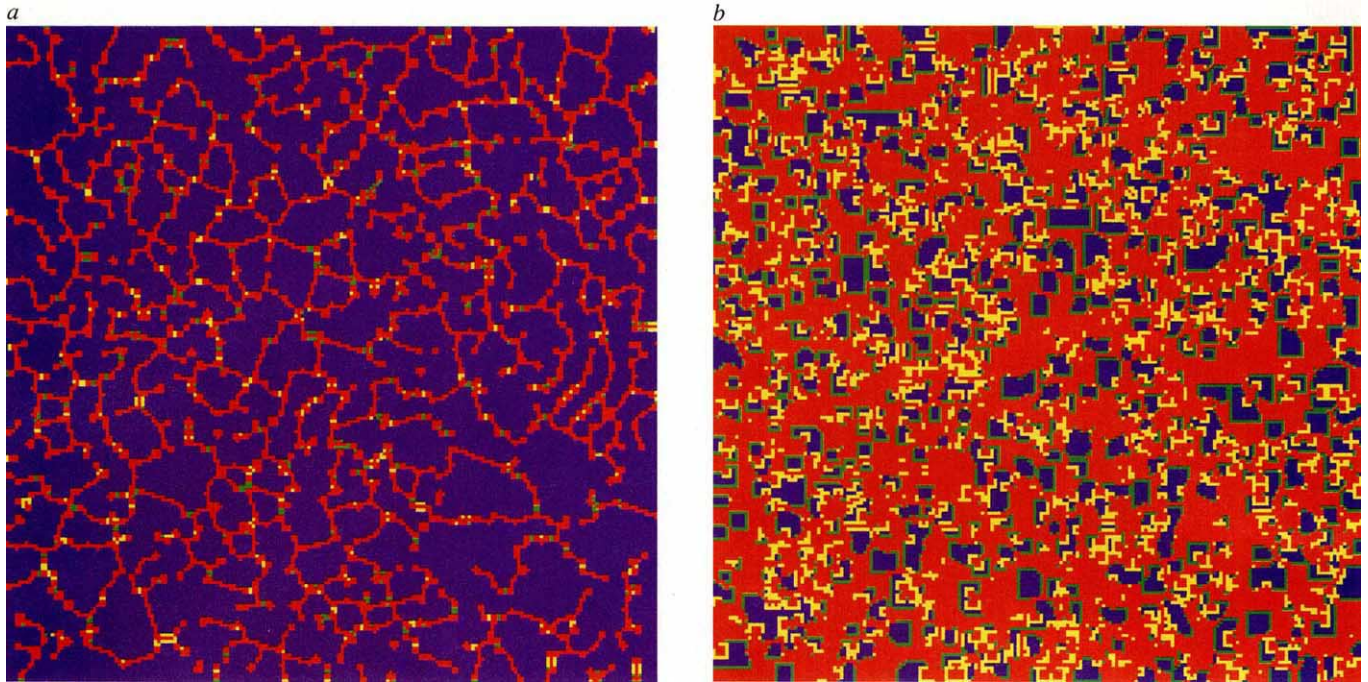
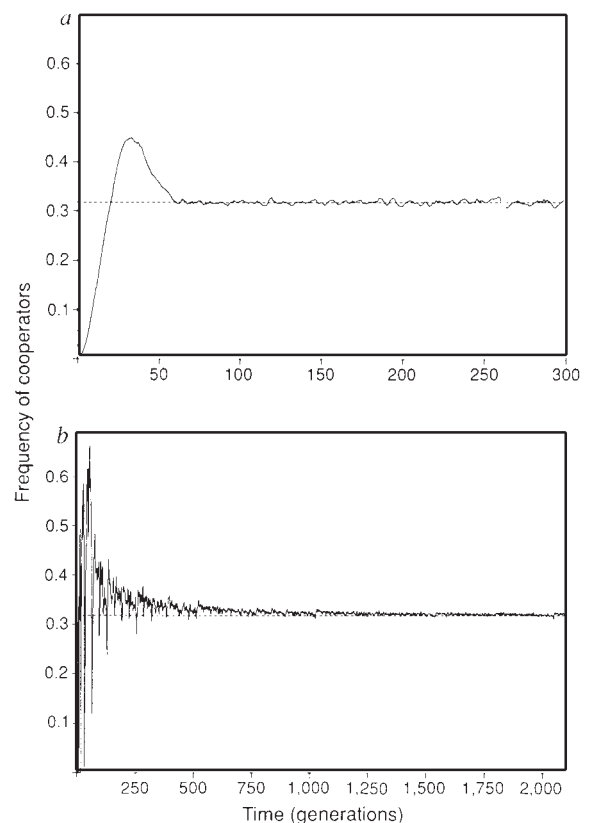


FIG. 1 The spatial Prisoners' Dilemma can generate a large variety of qualitatively different patterns, depending on the magnitude of the parameter, b , which represents the advantage for defectors. This figure shows two examples. Both simulations are performed on a 200×200 square lattice with fixed boundary conditions, and start with the same random initial configuration with 10% defectors (and 90% cooperators). The asymptotic pattern after 200 generations is shown. The colour coding is as follows: blue represents a cooperator (C) that was already a C in the preceding generation; red is a defector (D) following a D; yellow a D following a C; green a C following a D. *a*, An irregular, but static pattern (mainly of interlaced

networks) emerges if $1.75 < b < 1.8$. The equilibrium frequency of C depends on the initial conditions, but is usually between 0.7 and 0.95. For lower b values (provided $b > \frac{9}{8}$), D persists as line fragments less connected than shown here, or as scattered small oscillators ('D-blinkers'). *b*, Spatial chaos characterizes the region $1.8 < b < 2$. The large proportion of yellow and green indicates many changes from one generation to the next. Here, as outlined in the text, 2×2 or bigger C clusters can invade D regions, and vice versa. C and D coexist indefinitely in a chaotically shifting balance, with the frequency of C being (almost) completely independent of the initial conditions at ~ 0.318 .

FIG. 2 The frequency of cooperators in simulations with random or symmetrical initial conditions, within the interesting region $1.8 < b < 2$. *a*, The frequency of cooperators, $f_C(t)$, for 300 generations, starting with a random initial configuration of $f_C(0) = 0.6$. The simulation is performed on a 400×400 square lattice with fixed boundary conditions, and each player interacts with 9 neighbours (including self). The dashed line represents $f_C = 12 \log 2 - 8$ (see *b*). *b*, The frequency of C within the dynamic fractal generated by a single D invading an infinite array of C. At generation t , the width for the growing D structure is $2t + 1$, and the frequency of C, $f_C(t)$ within the square of size $(2t + 1)^2$ centred on the initial D site is shown as a function of t . This curve has interesting properties, which can be partly understood by referring to the geometry of the D structure. The D structures are closed-boundary squares in generations that are powers of 2, $t = 2^n$; hence $f_C(t)$ has minima at generations that are powers of 2. These squares now expand at the corners and erode along the sides, returning to square shape after another doubling of total generations. On this basis, a crude approximation suggests that, i time steps going from t to $2t$, there will be roughly $4(2i)/(2t + 1 - 2i)$ C sites within the D structure of size $(2t + 1 + 2i)^2$; hence the asymptotic C fraction, f_C , for very large such symmetrical patterns is $f_C = 4 \int_0^1 s(1-s)/(1+s)^2 ds = 12 \log 2 - 8 = 0.318 \dots$. As discussed in the text, this approximation agrees with the numerical results surprisingly well in both parts *a* and *b* of the figure.



fraction, f_C , shown in Fig. 2a is found for essentially all starting proportions and configurations for these b values.

Figure 3 is perhaps more in the realm of aesthetics than biology. Again $2 > b > 1.8$, but now we begin ($t = 0$) with a single D at the centre of a 99×99 lattice of Cs. Figure 3a shows the consequent symmetrical pattern 30 time-steps later, and Fig. 3b, c and d shows three successive patterns at $t = 217$, 219 and 221 after the pattern has reached the boundary (which happens at $t = 49$). These patterns, each of which can be characterized in fractal terms, continue to change from step to step, unfolding a remarkable sequence, dynamic fractals. The patterns show

every lace doily, rose window or Persian carpet you can imagine. As Fig. 2b shows, the asymptotic fraction of C is as for the chaotic pattern typified by Figs 1b and 2a. Many of the dynamic features of the symmetric patterns illustrated in Fig. 3 can be understood analytically. In particular, we can make a crude estimate of the asymptotic C fraction, f_C , for such very large symmetrical patterns. This approximation is shown by the dashed horizontal line in Fig. 2b, and it agrees with the numerical results significantly better than we would have expected. Why the approximation also works for the irregular, spatially chaotic patterns (Fig. 2a) we do not know.

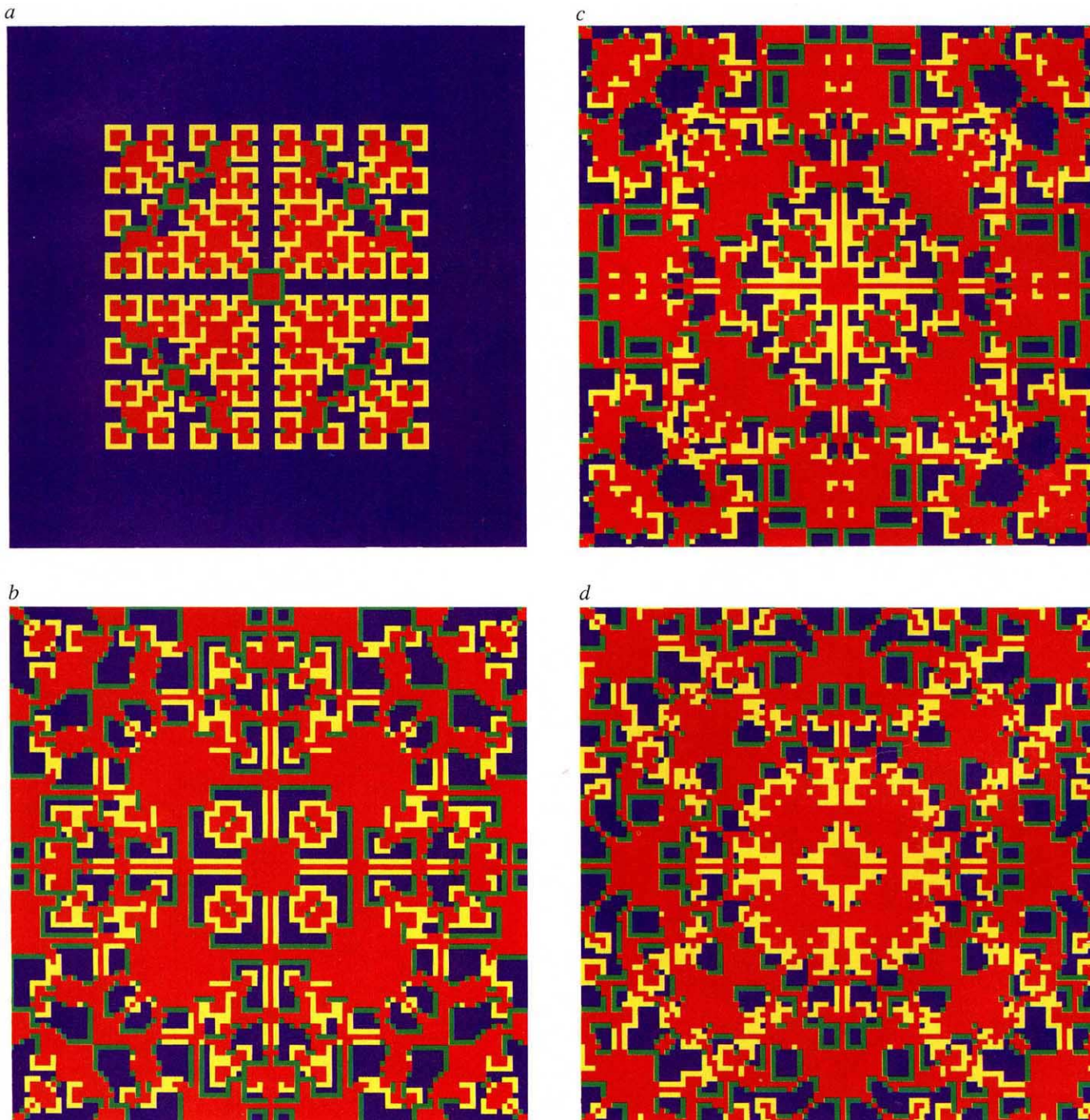


FIG. 3 Spatial games can generate an 'evolutionary kaleidoscope'. This simulation is started with a single D at the centre of a 99×99 square-lattice world of C with fixed boundary conditions. Again $1.8 < b < 2$. This generates an (almost) infinite sequence of different patterns. The initial symmetry is

always maintained, because the rules of the game are symmetrical. The frequency of C oscillates (chaotically) around a time average of $12 \log 2 - 8$ (of course). a, Generation $t = 30$; b, $t = 217$; c, $t = 219$; d, $t = 221$.

Our 'spatial dilemmas' game obviously invites comparison with more familiar cellular automata, such as Conway's 'Game of Life'⁸⁻¹¹. There are, however, qualitative differences. First, what happens to a site or cell in our lattice depends on the neighbours' scores, and thence on the state of the neighbours' neighbours. Thus, in the terminology of cellular automata, 25 cells are relevant to specifying the change in a given cell: the transition matrix has 2^{25} rules (this contrasts with Life, when 9 cells specify a cell's fate). That is, the motivating biological metaphor of the Prisoners' Dilemma generates a transition rule that is simple, but it would look horrendous if expressed in canonical cellular automata terms. Second, the patterns shown in Figs 1 and 3 have a combination of complexity and underlying regularity (exemplified by the asymptotic $f_C = 0.318$) unlike any cellular automata with which we are familiar. Third, we do also have a rich zoo of specific objects (rotators, gliders, blinkers, and an expanding jaw-like configuration of C cells that eats up a universe of D, leaving only structured strings of D ('eaters')) reminiscent of, but different from, those in Conway's Life^{8,9}. The taxonomy of this zoo is described elsewhere⁷.

Results similar to these are found if we exclude self-interaction, and consider interactions only with the eight nearest neighbours; here the 'interesting' region is $\frac{2}{3} > b > \frac{1}{3}$. The symmetrical patterns analogous to Fig. 3 are similarly kaleidoscopic, though different. The asymptotic C fraction, f_C , is now ~ 0.299 for both symmetric and random starting conditions. For interactions only with the four orthogonal neighbours, again the same qualitative regimes are found (here the interesting regime is $2 > b > \frac{3}{2}$ if self-interaction is included, and $\frac{3}{2} > b > \frac{4}{3}$ if not). Numerical studies suggest that f_C is around 0.374. Hexagonal arrays give complex patterns, but show less of the lacy, fractal character seen above, unless we weight the pay-offs from self-interactions somewhat more heavily than from neighbours (which is biologically plausible). In short, the above results seem robust⁷.

More generally, we have explored other evolutionary games played with neighbours in spatial lattices along the basic lines laid down above. They have features, particularly chaotic polymorphisms, similar to those seen for the spatial Prisoners' Dilemma. The hawk-dove game¹² gives notably beautiful patterns when begun from one hawk (or dove) invading an infinite array of doves (or hawks).

The Prisoners' Dilemma is an interesting metaphor for the fundamental biological problem of how cooperative behaviour may evolve and be maintained; alternative approaches involve, for example, studies of how the patchiness that can be created by limited dispersal or population 'viscosity' might favour the evolution of altruism through the elevation of inclusive fitness within kin groups¹³⁻¹⁵. Essentially all previous studies of the Prisoners' Dilemma are confined to individuals or organized groups who can remember past encounters, who have high probabilities of future encounters (with little discounting of future pay-offs), and who use these facts to underpin more-or-less elaborate strategies of cooperation or defection. The range of real organisms obeying these constraints is limited (although there is evidence suggesting 'tit-for-tat' strategies among some fish, birds, bats and monkeys^{5,6,16-18}). In contrast, our models involve no memory and no strategies: the players are pure C or pure D. Deterministic interaction with immediate neighbours in a two-dimensional spatial lattice, with success (site, territory) going each generation to the local winner, is sufficient to generate astonishingly complex and spatially chaotic patterns in which cooperation and defection persist indefinitely. The details of the patterns depend on the magnitude of the advantage accruing to defectors (the value of b), but a wide range of values leads to chaotic patterns, whose nature is almost always independent of the initial proportions of C and D. We believe that deterministically generated spatial structure within populations may often be crucial for the evolution of cooperation, whether it be among molecules, cells or organisms^{13-15,19-21}. Other evolutionary

games (hawk-dove, and so on) which recognize such chaotic or patterned spatial structure may be more robust and widely applicable than those that do not. More generally, such self-generated and complex spatial structures may be relevant to the dynamics of a wide variety of spatially extended systems: Turing models, 2-state Ising models, and possibly pre-biotic evolution¹⁹⁻²¹ (where it seems increasingly likely that chemical reactions took place on surfaces, rather than in solutions).

Although the motive for this work is primarily biological, we emphasize that 'spatial dilemmas' generate patterns of extreme richness and beauty. These have an aesthetic and mathematical (explaining, for example, why $12 \log 2 - 8$ in Fig. 2 works unexpectedly well) interest of their own. □

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Connectivity of chemosensory neurons is controlled by the gene *poxn* in *Drosophila*

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THE function of the nervous system depends on the formation of a net of appropriate connections, but little is known of the genetic program underlying this process. In *Drosophila* two genes that specify different types of sense organs have been identified: *cut* (*cr*)^{1,2}, which specifies the formation of external sense organs as opposed to chordotonal organs, and *pox-neuro* (*poxn*)³, which specifies the formation of poly-innervated (chemosensory) organs as opposed to mono-innervated (mechanosensory) organs. Whether these genes are also involved in specifying the connectivity of the corresponding neurons is not known. The larval sense organs are unsuitable for analysis of the axonal pathway and connections and so we have investigated the effect of *poxn* on the adult. Here we show that overexpression of *poxn* induces the morphological transformation of mechanosensory into chemosensory bristles on the legs and that the neurons innervating the morphologically transformed bristles follow pathways and establish connections that are appropriate for chemosensory bristles.

TABLE 1 Transformation of mechanosensory bristles into chemosensory bristles under different heat-shock conditions

Age of pupae (h APF)	T(°C)	t (min)	n (s.d.)
6	37	30	19.9 (0.0)
8	37	30	20.2 (4.4)
9	37	30	22.5 (3.8)
10	37	30	35.7 (7.0)
12	37	30	33.9 (5.1)
6	37	15	11.9 (3.4)
8	37	15	19.3 (2.6)
9	37	15	22.0 (1.9)
10	37	15	22.9 (4.9)
12	37	15	24.3 (3.0)
10	37	30	35.7 (7.0)
10	37	25	31.6 (6.2)
10	37	20	28.2 (5.5)
10	37	15	22.9 (4.9)
10	36	30	27.8 (3.4)
10	36	20	26.3 (3.8)
10	35	30	22.0 (4.4)

We used a transformant strain with a *hsp70-poxn* construct³ on the third chromosome. White prepupae (0 h APF) were left for the indicated times at 25 °C, transferred into a small basket and heat-shocked by immersion in a water bath at the indicated temperature, and left to develop at 22 °C. A heat shock of 30 min at 37 °C on a 10 h APF wild-type pupa has no effect on the morphology of the bristle ($n=8.0$); n is the mean number of chemosensory bristles on a mesothoracic tibia.

The legs of adult flies bear two types of sensory bristles (Fig. 1a). The first type are straight, pointed, bracted bristles innervated by a single neuron which responds to a deflection of the shaft, and therefore mediates touch (mechanosensory bristles). The second type are recurved, open-tipped, bractless bristles innervated by 2–4 neurons which respond to chemical stimulation of the dendrite and therefore mediate taste (chemosensory bristles). Each bristle derives from a single progenitor cell, the sensory mother cell (SMC), which undergoes a fixed sequence of cell divisions to generate the cells that produce the external parts of the sense organ (bristle and socket) and the innervating neuron(s)⁴. In the case of mechanosensory bristles, the formation of a bract by a neighbouring epidermal cell is induced by the bristle cells⁵. On the mesothoracic tibia of wild-type flies, mechanosensory bristles are arranged in 10 longitudinal rows which comprise 8 to 15 evenly spaced bristles depending on the row; chemosensory bristles are arranged in two rows of 4 or 5 bristles⁶.

In larvae, the deletion of *poxn* results in the morphological transformation of chemosensory into mechanosensory organs; an overexpression of the same gene induces the reciprocal transformation³. We first determined whether mechanosensory bristles of the adult legs can be morphologically transformed into chemosensory bristles as a result of ectopic expression of *poxn*, as was reported for larval sense organs. Generalized expression of *poxn* was obtained by heat-shocking transformant flies containing a construct with the coding region of the *poxn* gene under the control of a heat-inducible promoter (*hsp70-poxn*). Three strains containing the *hsp70-poxn* construct inserted at different locations have been tested for their response to heat shocks; all three showed a morphological transformation of larval mechanosensory into chemosensory organs (ref. 3; and C.D.C., unpublished observations). One of these strains was chosen for the analysis of neuronal connectivity. Because in the case of the wing disc, *poxn* is normally expressed in the SMCs of poly-innervated organs³, we heat-shocked pupae at the time of appearance of the SMCs of the mechanosensory bristles, between 6 and 12 h after puparium formation (APF). The SMCs of the chemosensory bristles appear earlier, between 4 and 6 h APF (refs 3 and 7; and K. I. Kimura, personal communication).

TABLE 2 Behavioural response of transformed bristles to stimulation

Bristle	Fly 1	Fly 2	Fly 3	Fly 4	Fly 5	Fly 6	Fly 7
A	+	+	+	+	+	+	+
B	+				+	+	+
C	+					+	
D	–				+		
A'	+						
B'	+					+	
C'	+						
D'	+				+		+
1		+	+	+			
2	–				+	+	
3	+		+			+	
4				+			
5		+	+		+		
6		+					
7					+		
11							+
12						+	
13				+			
14	+			+			
15		+	+				
16					+		
17					+	+	
18					+	+	
19			+				
21						+	
22						+	
23		+					
24	+	+		–			
25	+	+	+				+
26						+	
27							+

All flies were heat-shocked at 10 h APF, either for 20 min at 36 °C (individuals 1–4) or for 20 min at 37 °C (5–7) and left to develop at 22 °C until hatching. Flies were left in an empty vial overnight (~16 h) at 27 °C for wild-type flies and at 18 °C for *hsp70-poxn* flies. Every 30 min, each fly was tested by capping successively two mechanosensory bristles and chemosensory bristle A (see below) with a water-filled glass micropipette under $\times 200$ magnification (Nikon SMZ-U stereomicroscope). Flies showing spontaneous proboscis extension, or extension on stimulation of a mechanosensory bristle, or no extension on stimulation of the chemosensory bristle A, were retested at later times. Wild-type flies that respond to stimulation of bristle A generally respond to stimulation of all other chemosensory bristles (88 bristles tested in 11 individuals), but not to stimulation of mechanosensory bristles. Heat-shocked *hsp70-poxn* flies gave the response shown. By stimulating alternatively mechanosensory and transformed bristles of *hsp70-poxn* flies, we made sure that flies extended their proboscis only when a transformed bristle was stimulated (25 transformed bristles and 22 mechanosensory bristles tested in 7 individuals). Plus sign represents full extension of the proboscis upon stimulation; a minus sign represents no extension or partial extension after more than 20 s. A to D are wild-type chemosensory bristles of the posterior row; A' to D' are wild-type chemosensory bristles of the anterior row; 1 to 7 are transformed bristles of the row posterior to A–D; 11 to 19 are transformed bristles of the row between A–D and A'–D'; 21 to 27 are transformed bristles of the row anterior to A'–D'. Bristle numbering runs from distal to proximal part of the tibia, thus bristle 15 is always the fifth bristle of the second row and will occupy approximately the same position in all flies.

Heat shocks at 37 °C for 30 min of 10-h-APF wild-type pupae alter the alignment of bristles but have no effect on the appearance of external sense organs. Heat shocks of the *hsp70-poxn* transformant flies at 37 °C, 36 °C and 35 °C for 15 min and 30 min between 6 and 12 h APF induce a morphological transformation of mechanosensory into chemosensory bristles (Table 1 and Fig. 1b). We do not know whether the number of neurons innervating the morphologically transformed bristles is also typical of chemosensory organs. The maximal effect is obtained at 10 h APF with a heat shock of 30 min at 37 °C. In these conditions, up to 4 rows of mechanosensory bristles are transformed into

chemosensory bristles (Fig. 1b) and the mean number of chemosensory bristles reaches 36 instead of 9.

To test whether the neurons that innervate morphologically transformed bristles are also transformed, we first studied the normal axonal pathways in the central nervous system. Mechanosensory neurons follow either an anterior or a posterior

pathway, depending on the position of the bristle over the circumference of the leg⁸ (Fig. 2a and b). The posterior pathway extends along the posterior and medial periphery of the neuromere, the anterior pathway extends more laterally; the terminal arbors extend in the same focal plane and are not very branched. The projections of chemosensory neurons also follow either of two pathways, one anterior and one posterior, respectively followed by the neurons of the anterior row and by the neurons of the posterior row. Chemosensory projections can be distinguished from the mechanosensory projections by the property of the terminal arbor to penetrate deeply in the neuromere. In addition, anterior chemosensory projections can be distinguished from anterior mechanosensory projections by their extensively branched terminal arbor, whereas posterior chemosensory projections can be distinguished from posterior mechanosensory projection by their more central position in the neuromere. In order to establish the effect of a heat shock on

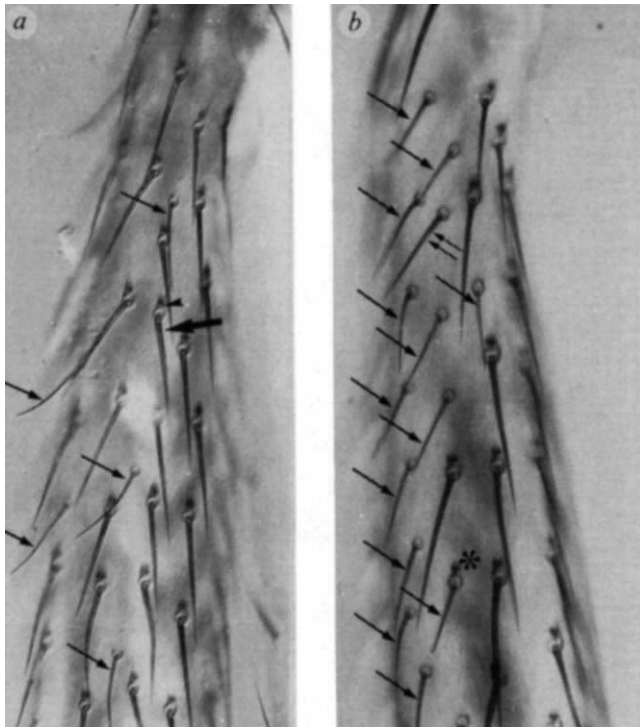


FIG. 1 Transformation of mechanosensory bristles into chemosensory bristles. Proximal region of the tibia of a, a wild-type fly and b, an *hsp70-poxn* fly heat-shocked at 10 h APF for 30 min at 37 °C. Mechanosensory bristles (thick arrow) have a sharp tip, chemosensory bristles (thin arrow in a) are thinner, have a blunt tip and tend to be recurved. In heat-shocked transformant flies, two to four rows of mechanosensory bristles near the normal chemosensory bristles are transformed (thin arrows in b). These bristles now assume the blunt-tipped morphology but are usually smaller and less recurved than normal chemosensory bristles, possibly because the mother cells of the normal chemosensory bristles (double arrow in b) emerge earlier. Mechanosensory bristles but not chemosensory bristles induce the differentiation of a cell, the bract (arrowhead in a); transformed bristles usually lose the ability to induce a bract; occasionally, however, a bract is formed (asterisk). Transformation was confined to the four rows of mechanosensory bristles near the normal chemosensory rows; this could be due to regional difference in the time of emergence of the mother cells, or in the sensitivity to *poxn* expression.

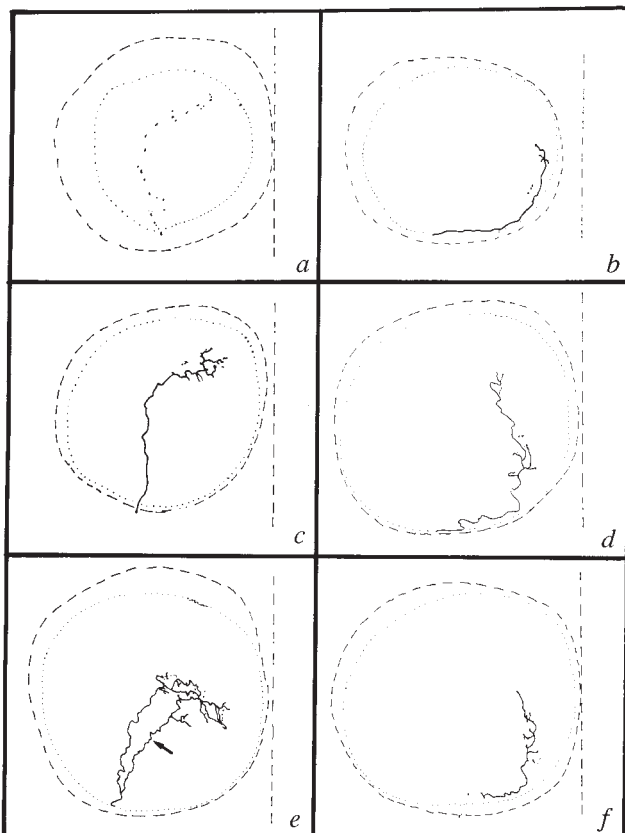


FIG. 2 Central projections from normal mechanosensory bristles (a, b), chemosensory bristles (c, d) and transformed bristles (e, f). a, Wild-type anterior mechanosensory bristle; b, wild-type posterior mechanosensory bristle. The anterior mechanosensory pathway extends ventrally; the terminal arbor is not branched and stays in the same focal plane than the projection. The chemosensory neurons following the anterior pathway extend also along the ventral side of the neuromere, but the terminal arbor penetrates deeply into the neuromere where it branches profusely. b, Posterior mechanosensory bristle and d, posterior chemosensory bristle in wild-type flies. The posterior mechanosensory pathway extends along the posterior and medial periphery of the neuromere. As in the case of the anterior mechanosensory projection, the terminal arbor is not branched and stays in the same focal plane than the projection. Conversely, the chemosensory neurons following the posterior pathway extend at the periphery of the neuromere for a short distance, then make a turn that brings them into the neuromere. At about a third of the pathway, they branch laterally then proceed to the anterior part of the neuromere. As in the case of the anterior chemosensory projection, the terminal arbor, and here, the lateral branches penetrate deeply in the neuromere. c, Projection of 1-3 anterior transformed bristles, the arrow indicates the oblique projection (see text); f, projection of 1-3 posterior transformed bristles; e and f were obtained in 10 h APF *hsp70-poxn* flies heat-shocked for 20 min at 37 °C. The projection from the small mechanosensory bristles of the thorax¹¹ and legs¹² of adult flies is often punctuated, as in a, possibly because of the small diameter of the axons. In many cases, the posterior projection is abortive and stops at the level of the lateral branch. The limit between transformed bristles that project along the anterior pathway and those that project along the posterior pathway lies between rows 5 and 6, and corresponds to the limit that determines, in the case of mechanosensory bristles, the type of pathway⁸. In all cases bristles of the right mesothoracic tibia were analysed. Sensory neurons were backfilled with horseradish peroxidase as described¹³, and filling times of 17 to 20 h at 18 °C were used. The dotted circle represents the limit of the leg neuromere at the focal plane of the projection, whereas the dashed circle represents the largest limit of the neuromere. The dashed vertical line represents the midline. In the horizontal view of the neuromere magnification is $\times 800$.

pathfinding in wild-type flies, we analysed the projection of mechanosensory bristles. In 11 out of 13 cases, the neurons established a wild-type projection, either of the anterior or the posterior type. But in two cases the axons followed an oblique pathway which was never observed in non-treated flies. In all cases, the terminal arbors are of the mechanosensory type. We then examined the projections established by the neurons innervating morphologically transformed bristles in heat-shocked *hsp70-poxn* flies. Neurons innervating transformed organs (Fig. 2e and f) can follow either of three pathways, the anterior and posterior chemosensory pathways and the oblique pathway that was also observed in heat-shocked wild-type flies. In all cases ($n=22$), independently of which pathway was followed, the terminal arbor penetrates deeply in the neuromere, as is typical of normal chemosensory axons. In most cases we filled 2 or 3 transformed bristles, and could not be sure whether the oblique projection is an independent projection, or a branch of the anterior projection. In one case, however, we observed that this pathway is actually a branch of the anterior pathway.

We designed a simple behavioural test to assess the connectivity of the transformed neurons. Stimulating a single leg bristle of a starved fly with a sugar solution is not sufficient to elicit proboscis extension⁹. But we found that thirsty flies do respond to stimulation with water of single chemosensory bristles (but not of mechanosensory bristles) by extending their proboscis. In our conditions, 26% of thirsty wild-type flies ($n=46$) respond to a stimulation of the chemosensory bristle A (Table 2). Whenever a fly gives a response to stimulation of bristle A, the stimulation of the other chemosensory bristles on the same tibia almost invariably induces an extension response (81 out of 88). When we tested the transformed bristles of heat-shocked *hsp70-poxn* flies ($n=36$; Table 2), we observed that flies that respond to the stimulation of bristle A almost

invariably respond to the stimulation of morphologically transformed bristles as well (38 out of 40).

In conclusion, we have shown that the ectopic expression of the *poxn* gene can induce a transformation of mechanosensory organs into chemosensory organs: the morphology of the transformed bristles is typical of chemosensory bristles, the bristle cells fail to induce the formation of a bract, the underlying neurons establish a projection that is clearly transformed towards that of normal chemosensory neurons, and they establish connections appropriate to chemosensory neurons. These results show that the *poxn* gene directs the choice of the axonal pathway and the establishment of connections by chemosensory neurons. As the *poxn* product contains a DNA-binding motif¹⁰ and is probably a transcriptional regulator, it seems likely that among its targets are genes that directly or indirectly encode the recognition of the appropriate axonal pathway and target neuron(s). □

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Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin

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CLOSTRIDIAL neurotoxins, including tetanus toxin and the seven serotypes of botulinum toxin (A–G), are produced as single chains and cleaved to generate toxins with two chains joined by a single disulphide bond (Fig. 1). The heavy chain (M_r 100,000 (100K)) is responsible for specific binding to neuronal cells and cell penetration of the light chain (50K), which blocks neurotransmitter release^{1–9}. Several lines of evidence have recently suggested that clostridial neurotoxins could be zinc endopeptidases^{2,10–14}. Here we show that tetanus and botulinum toxins serotype B are zinc endopeptidases, the activation of which requires reduction of the

interchain disulphide bond. The protease activity is localized on the light chain and is specific for synaptobrevin, an integral membrane protein of small synaptic vesicles. The rat synaptobrevin-2 isoform is cleaved by both neurotoxins at the same single site, the peptide bond Gln 76-Phe 77, but the isoform synaptobrevin-1, which has a valine at the corresponding position, is not cleaved. The blocking of neurotransmitter release of *Aplysia* neurons injected with tetanus toxin or botulinum toxin serotype B is substantially delayed by peptides containing the synaptobrevin-2 cleavage site. These results indicate that tetanus and botulinum B neurotoxins block neurotransmitter release by cleaving synaptobrevin-2, a protein that, on the basis of our results, seems to play a key part in neurotransmitter release.

To provide direct evidence that tetanus (TeTx) and botulinum (BoNT) toxins are zinc proteases and to identify their intracellular target(s) we used highly purified small synaptic vesicles from rat cerebral cortex^{15,16}, some protein components of which may be involved in neurotransmitter release^{17–19}.

Figure 1 shows that, out of the many proteins detected in highly purified small synaptic vesicles, only one protein band, with an electrophoretic mobility corresponding to that of synaptobrevin (19K)^{20–22}, was altered by incubation of the vesicles with TeTx. The intensity of the 19K band decreased with the concomitant appearance of 12K and 7K fragments. Like other bacterial protein toxins having intracellular targets, for example cholera and diphtheria toxins, both of which must be cleaved and reduced for activation²³, TeTx was active only in its double-chain reduced form.

In parallel experiments with BoNT serotype B (BoNT/B), which with serotypes A and E is most frequently involved in human botulism²⁴, the nicked and reduced BoNT/B specifically cleaved the 19K protein to give rise to fragments virtually identical to those produced by TeTx. BoNT/A and BoNT/E in

their reduced 2-chain form did not alter the small synaptic vesicle protein profile.

As would be expected for a zinc endopeptidase, EDTA blocks proteolysis by TeTx and BoNT/B. Moreover, captopril, a widely used antihypertensive drug that specifically inhibits the zinc-endopeptidase activity of angiotensin-converting enzyme²⁵, completely blocked the proteolytic activity of both TeTx and BoNT/B.

Cleavage of the 19K band did not exceed 60–70% (Fig. 2) and the time course of its proteolysis by TeTx and BoNT/B was comparable to the inhibition by these neurotoxins of neurotransmitter release in *Aplysia* neurons^{8,9} (see also Fig. 4).

Two synaptobrevin isoforms (SYB-1 and SYB-2; sequences shown in Fig. 3) have been identified in complementary DNA libraries from rat and human brain^{26,27}. Therefore the incomplete proteolysis of the 19K band shown in Figs 1 and 2 could be due to the selective action of the neurotoxins on one isoform. Figure 3 shows that under appropriate conditions the 19K band is indeed resolved into two components. Only the faster-migrating isoform is cleaved at a single site, as indicated by the size of the two proteolytic fragments, the absence of small peptides and the nearly quantitative conversion of the 19K band in the two fragments (Fig. 2).

To identify the target and site of action of TeTx and BoNT/B, we electroeluted and sequenced the 19K bands and fragments. The amino termini of the two 19K components and of the 12K fragment were found to be blocked. The 7K band produced by TeTx and by BoNT/B gave the same amino-acid sequence, FETSAAKLKRYWWKLNKMMILxV (in single-letter code; Fig. 3). This sequence corresponds to that of SYB-2 from residue 77 and identifies SYB-2 as the target of TeTx and BoNT/B proteolytic activity and the peptide bond Gln 76-Phe 77 as the site of cleavage. An identical target site for TeTx and BoNT/B within the nerve terminal, and another for BoNT/A and BoNT/E, explains earlier electrophysiological data^{5,7} and the different temperature sensitivity of these neurotoxins (B.P. *et al.*, manuscript in preparation), and is consistent with the sequence similarity between TeTx and BoNT/B, which is closer than that among other clostridial neurotoxins.

We cleaved the toxin-resistant slower-migrating 19K protein at the single Trp-Trp site with iodosobenzoic acid²⁸ and sequenced the fragments. One fragment was blocked but the other had the sequence xNxxMMIML, which confirmed its identity as SYB-1. This result explains the incomplete proteolysis of the 19K band shown in Figs 1 and 2, and indicates that SYB-1 and SYB-2 are present in our preparation of small synaptic vesicles

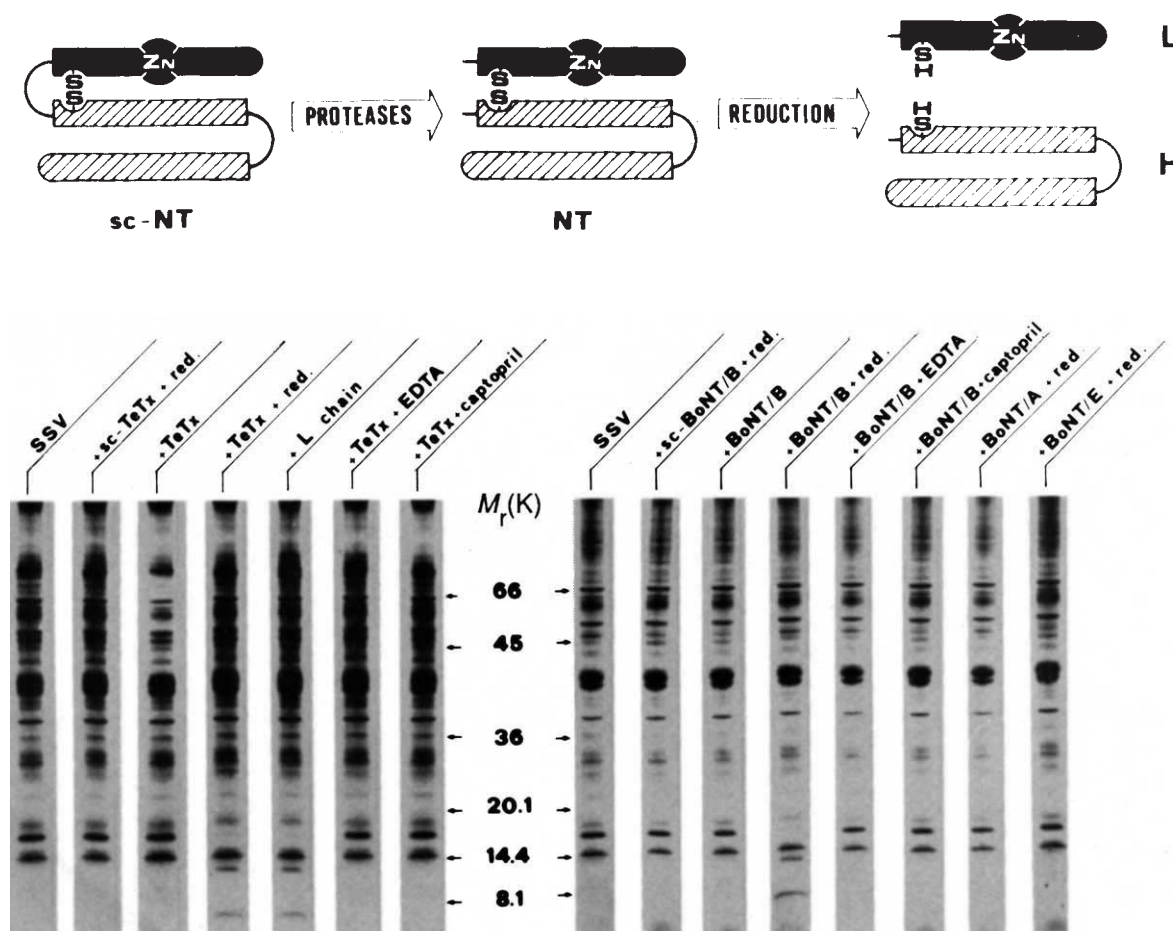
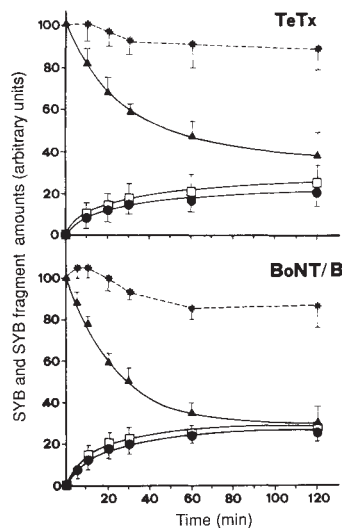


FIG. 1 Structure and proteolytic activity of tetanus and botulinum B neurotoxins on small synaptic vesicles. Upper panel, tetanus and botulinum neurotoxins are produced by bacteria as single chains and cleaved by different proteases at an exposed loop to generate the 2-chain toxin. The two chains can be separated by reduction of the single interchain disulphide bond³¹. H, heavy chain; L, light chain; sc-NT, single-chain neurotoxin; NT, neurotoxin. Lower left panel, 30 μ g small synaptic vesicles (SSV) in 5 mM HEPES-Na, 0.3 M glycine, 0.3 M NaCl, 0.02% Na₂S₂O₄, pH 7.4 (buffer A) were treated for 60 min at 37 °C with buffer (lane labelled SSV); with 200 ng single-chain TeTx reduced by incubation with 10 mM dithiothreitol (DTT) for 30 min at 37 °C (lane labelled sc-TeTx + red.); with 200 ng 2-chain TeTx

(TeTx); with 200 ng 2-chain TeTx reduced by incubation with 10 mM DTT for 30 min at 37 °C (TeTx + red.); with 200 ng TeTx L chain (L chain); with the same amount of reduced 2-chain TeTx in the presence of 10 mM EDTA (TeTx + EDTA) or of 1.4 mM captopril ([([2S]-1-[[3-mercapto-2-methylpropionyl]-L-proline]) (TeTx + captopril). Lower right panel shows results of similar treatment with BoNT/B of 30 μ g of SSV in buffer A, and in the last two right-hand lanes, of treatments with 200 ng reduced 2-chain BoNT/A (BoNT/A + red.), and 200 ng reduced di-chain BoNT/E (BoNT/E + red.). Samples were made 2% in SDS, 5 mM EDTA, boiled for 3 min, electrophoresed in a 12-cm-long 12–18% polyacrylamide gradient gel and stained with Coomassie blue²¹. Migration of M_r standards is shown in the centre.

FIG. 2 Time course of the proteolysis of the 19K protein (SYB) of small synaptic vesicles (SSV) induced by tetanus and botulinum B neurotoxins. SSV were incubated in buffer A as for Fig. 1 with 15 nM (final concentration) reduced 2-chain TeTx or 22 nM reduced 2-chain BoNT/B at 37 °C. At the times indicated, 30 µg SSV protein aliquots were withdrawn, quenched by addition of SDS-PAGE sample buffer, electrophoresed and stained as for Fig. 1. Gels were scanned with a dual-wavelength Shimadzu CS-930 densitometer and values of the integrated peaks at 19K (SYB, ▲); 12K (P12, ●) and 7K (P7, □) and of their sums (*) at various incubation times are shown. Bars represent s.d. of three different experiments.



in a ratio of ~1:2, which correlates with the amount of their respective messenger RNAs in rat brain²⁷. The resistance of rat SYB-1 is probably due to the presence of a valine residue at the position corresponding to the cleavage site of SYB-2, where SYB-2 has a glutamine (see later). Both human SYB-1 and SYB-2 are expected to be cleaved by the two neurotoxins, since both of them have a glutamine residue at the cleavage site.

If the specific proteolytic cleavage of SYB-2 is the cause of the inhibition by TeTx and BoNT/B of neurotransmitter release, then a peptide sequence encompassing the cleavage site should inhibit the intracellular poisoning that results when the two neurotoxins are injected into neurons of the buccal ganglion of *Aplysia californica*, a well characterized electrophysiological system⁸⁻¹⁰. Figure 4 shows that peptides ASQFETS and QFET, which contain the cleavage site of SYB-2, were very effective at delaying the inhibition induced by TeTx injected into *Aplysia* neurons. We obtained similar results with BoNT/B, but found that the same peptides were ineffective against BoNT/A and BoNT/E (data not shown). In our *in vitro* assay with small synaptic vesicles, QFET completely prevented proteolysis of

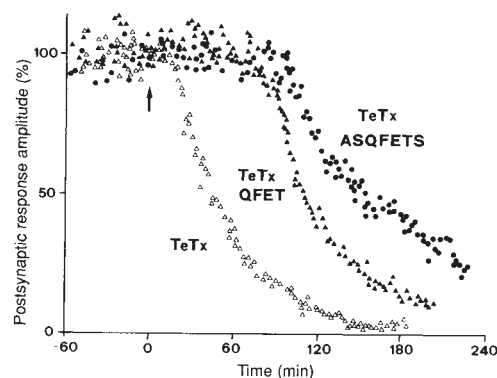


FIG. 4 The inhibition of neurotransmitter release in *Aplysia* neurons injected with tetanus toxin is delayed by SYB-2 peptides ASQFETS and QFET. Release of acetylcholine at identified cholinergic couples of neurons of the buccal ganglion of *Aplysia californica* was evoked by a presynaptic action potential, and the postsynaptic response was recorded under voltage-clamp^{8,9}. After control recordings were made, presynaptic neurons were injected (arrow) with TeTx (intracellular concentration 8 nM) or with TeTx preincubated with ASQFETS or QFET (intracellular concentration 80 µM). Injection of buffer or peptides had no effect on acetylcholine release (not shown).

SYB-2 by TeTx or BoNT/B. It is notable that no protein in the sequence data banks apart from SYB-2 contains the ASQFETS sequence.

Features already discussed¹⁷⁻¹⁹ of the complex process of neurotransmitter release are now becoming clearer. Recent evidence implicates synaptophysin and synaptotagmin in neurotransmitter release^{29,30}. So far no function has been assigned to synaptobrevins. Our findings indicate that SYB-2, a protein highly conserved during evolution²², plays a fundamental but as yet undetermined role in the neuroexocytotic process. Perhaps cleavage of SYB-2 alters the recognition of a complementary binding protein present in the cytosol or on the cytoplasmic face of the plasma membrane, so impairing neurotransmitter vesicle fusion.

This SYB-2 specific zinc-endopeptidase activity of TeTx and BoNT/B offers an experimental route to treatment of the two widespread neuroparalytic disorders tetanus and botulism. When a specific inhibitor of zinc endopeptidases like captopril is injected into mice together with TeTx or BoNT/A or B, it prevents intoxication (O.R. *et al.*, unpublished results); it is

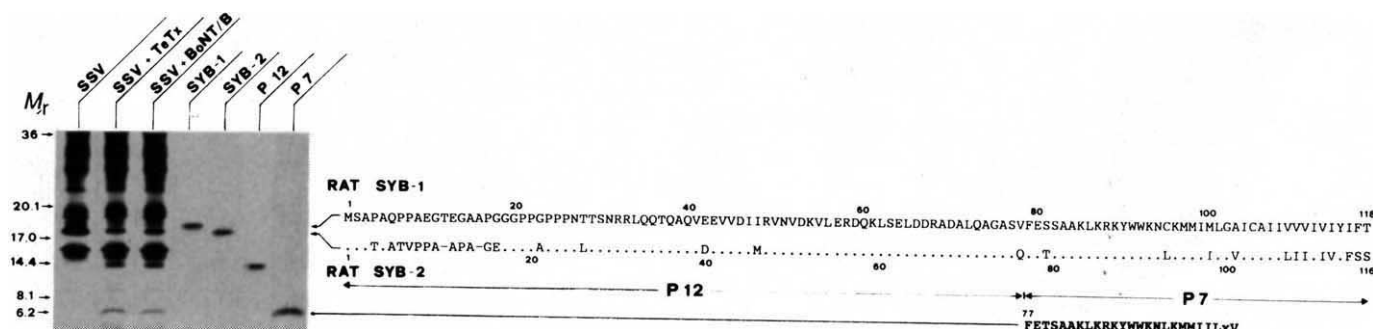


FIG. 3 Synaptobrevin-2 is specifically proteolysed by tetanus and botulinum B neurotoxins at peptide bond Gln 76-Phe 77. SSV protein (8 µg) was incubated as for Fig. 1 with buffer (lane SSV), with reduced 2-chain TeTx (50 ng; lane SSV + TeTx) or with BoNT/B (50 ng; SSV + BoNT/B) for 120 min at 37 °C, then electrophoresed in a 20-cm 12–18% polyacrylamide gradient gel and silver-stained. Loading of small amounts of SSV proteins was necessary to resolve the 19K band of Fig. 1 into a doublet. In the other gel lanes the doublet at 19K and the 12K and 7K fragments were sliced and the individual bands electroeluted. The eluted proteins proved to be single components after electrophoresis and silver staining (lanes SYB-1, SYB-2, P12 and P7).

Proteins were blotted onto Pro-Blot and sequenced in a pulsed liquid Applied Biosystem Mod.477A protein sequencer. The two 19K and the 12K proteins did not give any signal, whereas the 7K fragment generated both by TeTx and BoNT/B gave the sequence FETSAAKLRKRYWKNLKMILKxV, which corresponds to SYB-2. A single cleavage site is also indicated by comparison of the SYB sequences, because the central region of the two SYBs is identical and additional cleavages must occur in this region to account for the size of the proteolytic products. Each sequencing cycle gave a single residue. From the ratio of the Thr and Ser peaks at the third cycle, it could be estimated that the extent of SYB-1 cleavage was always <4%.

therefore a promising possibility that some zinc endopeptidase inhibitors could eventually be useful in the clinical treatment of tetanus and botulism. □

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Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene

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MURINE *Hox* genes have been postulated to play a role in patterning of the embryonic body plan^{1–3}. Gene disruption studies have suggested that for a given *Hox* complex, patterning of cell identity along the antero-posterior axis is directed by the more 'posterior' (having a more posterior rostral boundary of expression) *Hox* proteins expressed in a given cell^{4–6}. This supports the 'posterior prevalence' model², which also predicts that ectopic expression of a given *Hox* gene would result in altered structure only in regions anterior to its normal domain of expression. To test this model further, we have expressed the *Hox-4.2* gene more rostrally than its normal mesoderm anterior boundary of expression, which is at the level of the first cervical somites. This ectopic expression results in a homeotic transformation of the occipital bones towards a more posterior phenotype into structures that resemble cervical vertebrae, whereas it has no effect in regions that normally express *Hox-4.2*. These results are similar to the homeotic posteriorization phenomenon generated in *Drosophila* by ectopic expression of genes of the homeotic complex HOM-C (refs 7–10; reviewed in ref. 3).

A wild-type *Hox-4.2* complementary DNA, and two mutant cDNA forms that show greater transcriptional transactivation in transfected tissue culture cells (C.H. and P.C., unpublished results) were placed under the control of the *Hox-1.6* promoter (Fig. 1a). This promoter is active in the rostral hindbrain and in paraxial mesoderm from an anterior boundary of expression rostral to the occipital somites extending caudally to the mid-thoracic somites, with expression first detectable from a *LacZ* (β -galactosidase) construct at day 7.5 and continuing until day 12.5. (T.L., M.L. and P.C. manuscript in preparation; Fig. 1b). Transgenic mice bearing these constructions were delivered by caesarean section at day 10.5 and day 18.5 of gestation, and identified by Southern analysis of yolk sac or placental DNA taken from each newborn. Ten transgenic embryos at day 10.5 were analysed by RNA *in situ* hybridization for ectopic expression of the *Hox-4.2* transgene, as well as for expression of the endogenous paralogue, *Hox-1.4* (Fig. 1b). *Hox-4.2* transcripts were detected in a more rostral domain in the transgenic embryos compared with wild-type embryos (Fig. 1b legend). Interestingly, there was no detectable effect of ectopic *Hox-4.2* expression on the domain of expression of the paralogous gene *Hox-1.4* (Fig. 1b). Out of 32 day-18.5 transgenic animals, 19 were stillborn with many abnormalities (Figs 2 and 3). The remaining transgenics were viable and phenotypically normal, probably owing to inactivation of the transgene upon integration.

Examination of the skeletons of both transgenic and non-transgenics revealed a number of abnormalities observed only in the transgenic newborns, although the severity varied between individuals (Figs 2 and 3, and data not shown; see Fig. 4 for summary). The most striking abnormality initially observed was the absence or reduction in size of the supraoccipital (S) and exoccipital (E) bones of the skull, and the presence of ectopic bony structures resembling additional neural arches located anterior to the first cervical vertebra, the atlas (C1), and fused ventrally to the basioccipital bone (B) (compare 'normals' in Fig. 2a and c with 'transgenics' in Fig. 2b, d, e and f; see Fig. 4 for a schematic representation of the defects in the braincase and cervical region). The number of ectopic neural arches observed in the transgenics ranged from one to four (Fig. 1a). The most dramatic phenotype showed four ectopic neural arches (E^+ in Fig. 2b and d). Transgenics with one or two ectopic neural arches (E^+) are shown in Fig. 2e and f, respectively, which is distinct from the exoccipital bone remnant (E^0 ; compare Fig. 2c with e and f). Note that in the case of weakly transformed animals (Fig. 2e), distinguishing between E^0 and E^+ is problematic. Severe modifications of the basioccipital (B) and basisphenoid (BS) bones were observed. This included ectopic neural arch-like ossifications (distinct from the exoccipital bone remnant, E^0) fused to the basioccipital bone (B, compare Fig. 2g and h), and partial or complete agenesis of the basisphenoid bone (BS, compare Fig. 2i and j).

Histological sections of both a transgenic and a normal newborn are shown in Fig. 3. This transgenic newborn displays a phenotype similar to the one in Fig. 2e, with a single ectopic neural arch (E^+ ; white arrow in Fig. 3c, compare with d). Characteristics common to all phenotypically abnormal transgenic newborns were as follows. They have altered exoccipital bones (E^0 ; compare Fig. 3a, c and e with b, d, and f), and one to four ectopic neural arches (E^+ ; compare Fig. 3c and d). The occipital condyles are lacking (CO; compare Fig. 3a and b) resulting in the absence of the joint (atlanto-occipital joint) between the exoccipital bone and the arch of the first cervical vertebra, the atlas (C1A). The second cervical vertebra, the axis (C2), is devoid of a dens (D) in the transgenics (compare Fig. 3c and d), because the atlas-axis (atloaxoid) vertebral body (C1-2) is fused with the basioccipital bone (B), which itself contains a notochord remnant (NT), and has assumed a cylindrical shape, similar to a vertebral body (compare Fig. 3c and e with d and f). The basisphenoid bone (BS) is aplastic or

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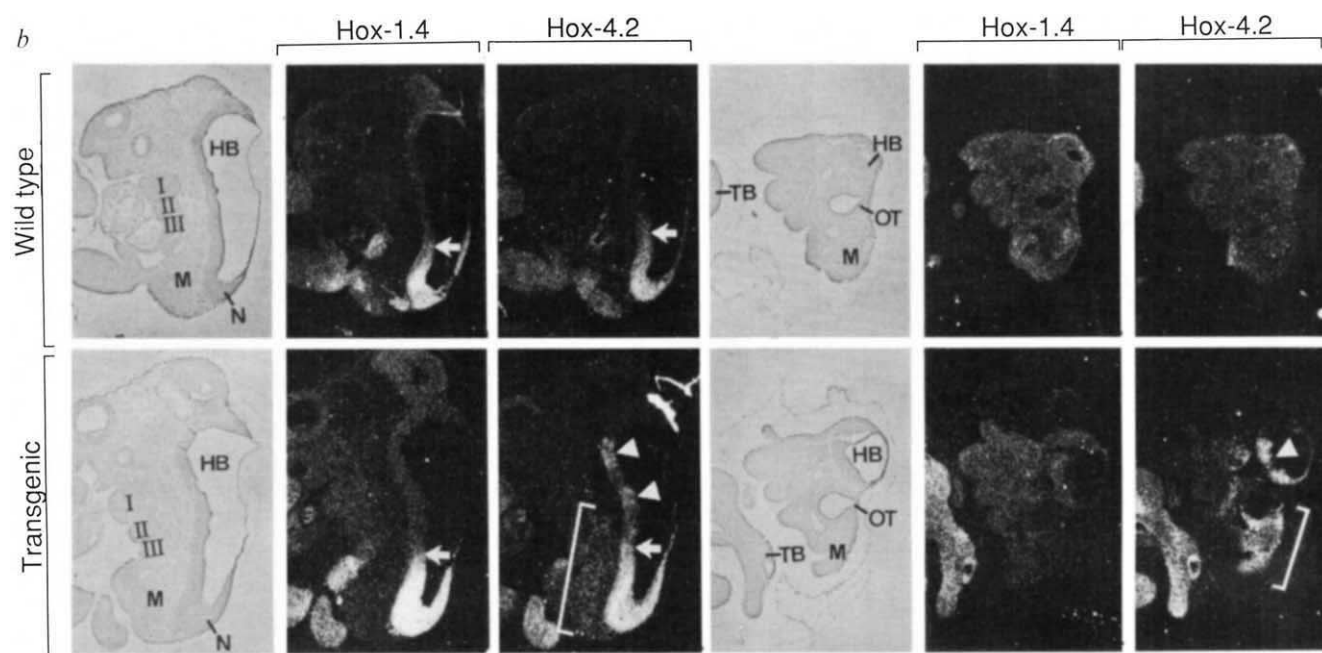


FIG. 1 *a*, Structure of the *Hox-4.2* ectopic expression constructs, tabulation of their transcription activation in HeLa cells, the number of phenotypically abnormal transgenic animals obtained, and the number of ectopic neural arches observed with each construct. The *Hox-1.6* promoter from -7 kilobase pairs (kb) to +40 base pairs (bp) (relative to the transcription initiation site) was placed upstream of the wild type and two mutant forms of the *Hox-4.2* cDNA, followed by the SV40 polyadenylation signal. The fold increase of each *Hox-4.2* cDNA on transcription in transfected tissue culture cells (relative to no cDNA transfected) is shown. The range of ectopic neural arches seen with each construct is indicated. *b*, RNA *in situ* analysis of *Hox-1.4* and *Hox-4.2* mRNA distribution in a wild type (top panels) and transgenic (bottom panels) embryo at day-10.5 post coitum. For each embryo, two representative sagittal sections are shown: a medial section crossing the hindbrain (HB) and branchial arches (left panels) and lateral sections crossing the otocyst (OT) (right panels). *In situ* hybridization signals appear white on the dark-field photographs. In the wild-type embryo, *Hox-1.4* and *Hox-4.2* transcript domains are very similar both in neural and mesenchymal tissues. Note there is no significant labelling in the lateral sections (right panels). There is no apparent alteration of *Hox-1.4* transcript distribution in the transgenic embryo. In contrast, *Hox-4.2* transcripts are detected which extend more anteriorly than the 'endogenous' domain both in neurectoderm (with two distinctly labelled areas (white triangles) corresponding to rhombomeres 2 and 4) and in the head mesenchyme (white brackets). In particular, *Hox-4.2* transcripts extend widely beyond its normal boundary (white brackets), both in medial (sclerotomal) and lateral head mesenchyme, up to the otocyst. White arrows point to the respective 'endogenous' *Hox-1.4* and *Hox-4.2* transcript boundaries in the hindbrain. White brackets indicate the mesenchymal area, which is labelled by the *Hox-4.2* probe exclusively in the transgenic embryo, and which is located more rostral than the endogenous *Hox-4.2* transcripts in mesenchyme. Abbreviations: HB, hindbrain; M, mesenchyme; N, neurectoderm; OT, otocyst; TB, Tail bud; I, II, III, branchial arches I, II, and III respectively.

METHODS. The *Hox-1.6* gene containing plasmid p313.2 (ref. 5) was modified by conversion of the restriction enzyme *Bam*HI cutting site at +40 bp (relative to the transcription initiation site at +1) to an *Xba*I site by the addition of an oligonucleotide adaptor to generate plasmid p649. From plasmid p649 the 7-kb *Eco*RI-*Xba*I fragment spanning from -7 kb to +40 bp was subcloned into the *Eco*RI-*Xba*I site of pPolyIII (ref. 23) to generate plasmid p777. The 175-bp *Xho*I fragment containing the SV40 polyadenylation signal from pTL-2 was inserted into the unique *Sal*I site of p777 to generate p847-4. pTL-2 was constructed by inserting an oligonucleotide containing the additional restriction sites (*Not*I-*Hind*III-*Sst*I-*Pst*I-*Xho*I-*Kpn*I) inserted into the *Eco*RI-*Bam*HI site of pSG5 (ref. 24). p847-4 was digested at the unique *Nru*I site located between the *Hox-1.6* promoter fragment and the SV40 polyadenylation signal, and the *Hox-4.2* cDNA forms were inserted. The *Hox-4.2* cDNA forms were constructed by combining two partial cDNA clones (ref. 25) at their common *Eco*RI site and then cloning into the *Bgl*II site of pSG4 (ref. 24). Mutant derivatives of the wild-type cDNA were made by oligo-mediated site-directed mutagenesis. An *Xho*I site was inserted at various locations to permit the construction of the cDNA forms shown. The 'truncated' cDNA form deletes all 39 amino acids on the carboxy side of the homeodomain, replacing them with Asn-Pro-Arg-Glu-STOP immediately following the His at amino-acid

<i>a</i>	Hox-1.6 promoter -7kb to +40bp	Hox-4.2 cDNA	SV40 poly-A site	Form of <i>Hox-4.2</i> cDNA	Fold stimulation of transcription in transfected HeLa cells	Stillborn transgenic animals	Ectopic neural arches
				Wild type (250AA)	70X	5	1-3
				Truncated (211AA)	300X	11	1-3
				Duplicated (AA6-81)	2900X	3	1-4

position 211. The 'duplicated' cDNA form contains the *Hox-4.2* protein sequence from amino acids 1 to 81, followed by a 3-amino-acid insertion (Pro-Arg-Glu) and then runs from amino acid 6 to the carboxy terminus at amino acid 250. Hence this cDNA form duplicates the region from amino acids 6 to 81. The *Hox-4.2* cDNA forms were excised by digestion with restriction enzymes *Sst*I and *Xho*I, ends were blunt-filled with Klenow enzyme, and cloned into the *Nru*I site of p847-4. The resulting plasmids p848a 'wild-type', p677 'truncated', and p678 'duplicated' were purified by banding twice in CsCl gradients. The *Hox-1.6* promoter-*Hox-4.2* cDNA insert was purified away from vector sequences by digestion with *Sfi*I restriction enzyme followed by purification on a sucrose gradient. The purified DNA fragment was resuspended in 5 mM Tris-HCl, pH 8, 0.5 mM EDTA, at a concentration of 2 ng μ l⁻¹ and used directly for microinjection as described²⁶. Transactivation analysis was assayed by co-transfection of the *Hox-4.2* cDNA forms with a reporter plasmid and a control plasmid in HeLa cells followed by quantitative S1 nuclease analysis. The reporter plasmid contained a homeodomain recognition sequence concatamer (four tandem copies of TCGGCCATTACCATTCATTAC) cloned upstream of the rabbit β -globin promoter at position -109 in pG1 (ref. 27). The SV40 promoter-driven plasmid pA56 served as a control for transfection efficiency²⁸; 5 μ g expression plasmid, 2 μ g reporter plasmid, 5 μ g control plasmid and 10 μ g carrier plasmid pSP65 (Promega) were transfected by the calcium phosphate co-precipitation technique into HeLa cells. RNA was isolated after 48 h and 10 μ g assayed by S1 nuclease analysis²⁷. Fold increase is calculated compared to transfected pSG4 without a *Hox-4.2* cDNA insert. Western analysis and *in vitro* DNA-binding analyses of total and nuclear proteins indicated that similar amounts of the *Hox-4.2* proteins were produced in HeLa cells. For RNA *in situ* hybridization, embryos were fixed overnight in 4% paraformaldehyde, dehydrated in graded ethanol, embedded in paraffin, sectioned in a sagittal plane, and hybridized with ³⁵S-labelled antisense RNA probes to *Hox-1.4* and *Hox-4.2* as described²⁹. Emulsion-coated sections were exposed for 3 weeks. Ten transgenic embryos were analysed, of which three showed a clear ectopic rostral expression of *Hox-4.2*. In all cases, *Hox-1.4* labelling was indistinguishable from that in wild-type (control) animals.

absent in the transgenics, consequently the hypophysis (H) always lies in direct contact with the pharynx (P) (compare Fig. 3g and h; see Fig. 4 for a schematic diagram of the defects). The palatal processes (PP) of the maxilla are unfused, resulting in the absence of the nasopharyngeal duct (NP) (compare Fig. 3i and j). The brain cavity in the transgenics is narrowed in the occipital region, and the cerebellum (C) appears to have grown inwards instead of expanding at the dorsolateral surface of the brain (small black arrows; compare Fig. 3k and l). In addition, the hindbrain (HB) appears to be compressed by the malformed basioccipital bone (large black arrow; compare Fig. 3k and l). The abnormalities in the palatal processes (PP), nasopharyngeal duct (NP), cerebellum (C), and hindbrain (HB) are likely to be secondary effects resulting from the mechanical stress induced by the altered axial skeleton described; but a direct effect of the transgene on these structures cannot be ruled out. Most importantly, no abnormalities were detected in transgenic newborns

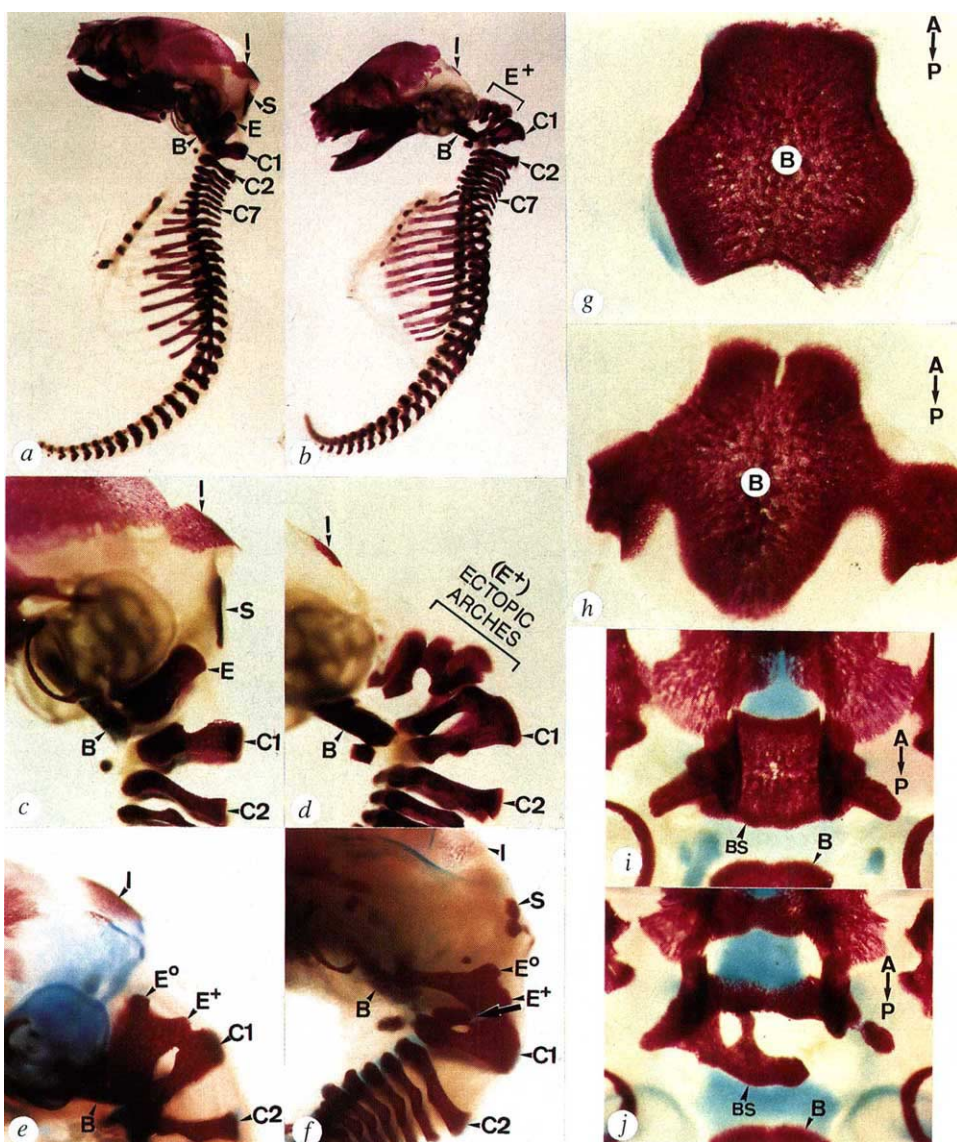
in regions where *Hox-4.2* is expressed posterior to the axis (C2) (compare, for example, Fig. 2a and c with b, d, e and f), although the transgene is expressed caudally to the level of the mid-thoracic somites (data not shown).

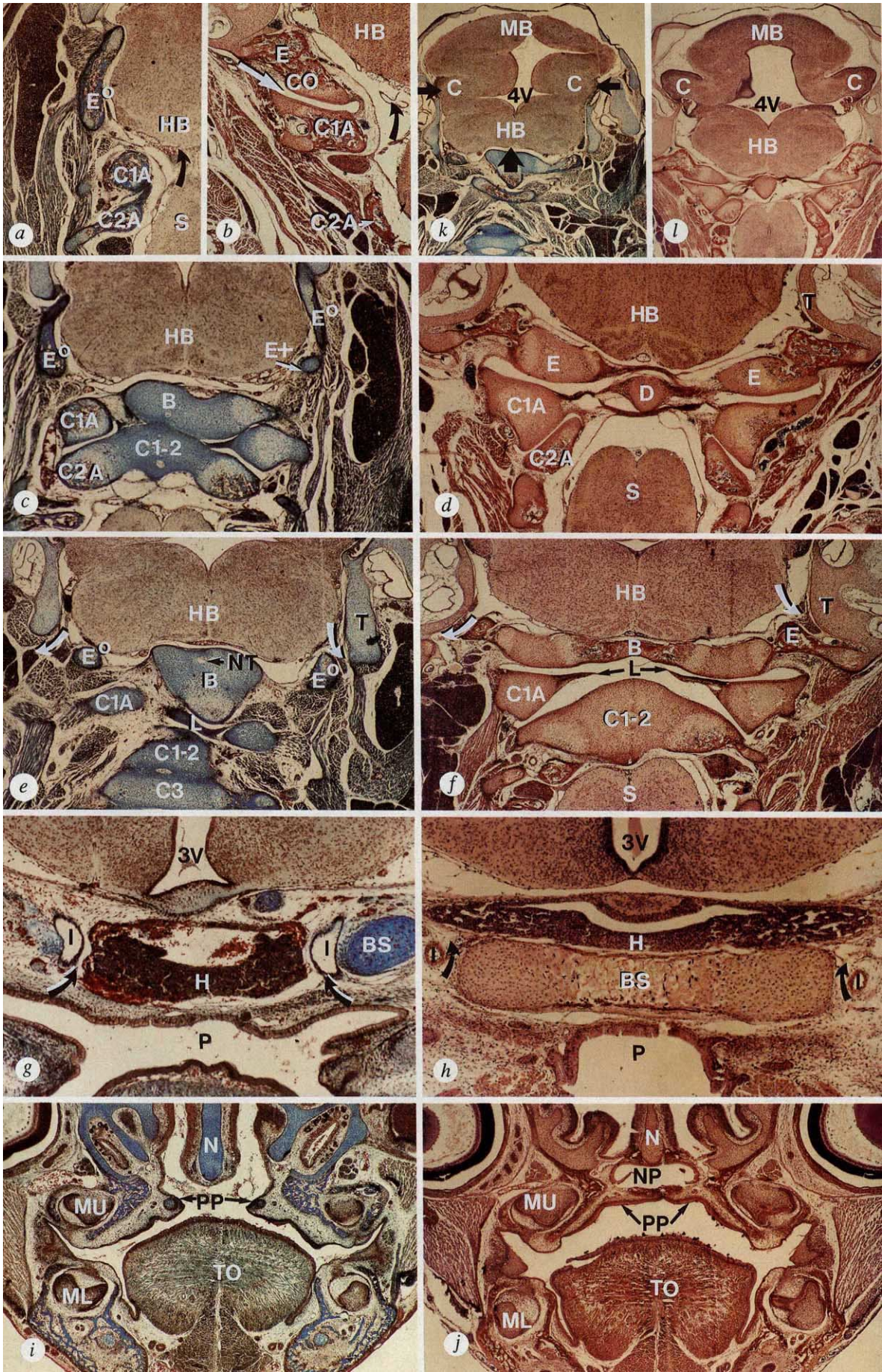
During normal development of the vertebral column, the sclerotome cells of each somite migrate towards the notochord and neural tube, where with the sclerotome cells of the adjacent somites, they merge to form an elongated sclerotomal column^{11,12}. Subsequently this column undergoes 'resegmentation' ('Neugliederung'¹³) to give rise to prevertebrae and intervertebral discs^{13,14}. The occipital region somites (O1–O4) differ from the developmental program of the rest of the vertebral column, as the sclerotomal column does not resegment in this region^{12,15}. The resulting basioccipital bone is the occipital equivalent of a vertebral body, and the exoccipital bone is the occipital equivalent of a neural arch, each derived from the fusion of the four occipital somites^{15,16} (O1–O4; see Fig. 4e,

FIG. 2 Whole-mount alizarin red-stained and alizarin red/alcian blue-stained skeletons of transgenic and non-transgenic (normal) newborns. Normal (a, c, g, i) or transgenic (b, d, e, f, h, j) alizarin red-stained (a, b, c, d) or alizarin red/alcian blue-stained (e, f, g, h, i, j) axial skeletons or individual parts viewed laterally (a–f), dorsally (g, h) or ventrally (i, j). A→P indicates the direction of the antero-posterior axis. a and b, Malformations observed in the transgenics. The reduction in size of the interparietal bone (I) and the complete absence of the supraoccipital bone (S) and exoccipital bone (E). The first cervical vertebra, the atlas (C1), the second cervical vertebra, the axis (C2), the seventh cervical vertebra (C7), and the basioccipital bone (B) are indicated. Ectopic neural arches (E⁺) are indicated. c–f, Occipital-cervical region of a normal newborn (c) and transgenic (d–f) newborns in more detail. Transgenics in e and f have a less dramatic phenotype than in b and d, displaying an exoccipital bone remnant (E⁰) and one or two ectopic neural arches (E⁺) respectively. c and d, Same animals as in a and b. Note the transformation in a–f of the supraoccipital (S) and exoccipital bones (E) into ectopic neural arches (E⁺), which are partially fused to one another and to the posterior arch of the atlas (C1). g and h, Transformation of the basioccipital bone (B), which has ectopic lateral ossifications distinct from the exoccipital bone remnant (E⁰; e and f). i, j, Aplasia of the basisphenoid bone (BS) in skulls stained with both alcian blue and alizarin red; the position of the basioccipital bone (B) is indicated.

METHODS. Newborns were delivered by caesarean section at day 18.5 of gestation. DNA was extracted from a tissue sample and analysed by southern blotting to determine the presence of the transgene as described⁵. Alizarin red-staining of bones was as described⁵. For combined alcian blue-staining of cartilage and alizarin red-staining of bone, after overnight fixation in 95% ethanol, each skeleton was incubated in one volume of glacial acetic acid, four volumes 95% ethanol and 7.5 mg alcian blue (Sigma) in 50 ml final volume. After 24–48 h, this solution was removed and the skeletons were rinsed in 95% ethanol for 1 h and the ethanol

replaced with 2% KOH. After 6–24 h, the 2% KOH was replaced with 1% KOH and 75 µg ml⁻¹ alizarin red S (Sigma) for 12–24 h. Skeletons were then cleared in 20% glycerol, 1% KOH for a week, with the solution being changed daily. Skeletons were then transferred to 50% glycerol, 50% ethanol for photography and storage.





normal, and Fig. 4a). In the stillborn transgenics ectopically expressing *Hox-4.2*, the occipital bones are transformed towards a phenotype that resembles cervical vertebrae. The fusion of the axis dens (D) (which is normally the most anterior tip of the vertebral column) to the basioccipital bone extends the fusion which is characteristic of the atloaxoid (C1-2) vertebral body into the occipital region (compare normal with transgenic vertebral body in Fig. 4e). In normal mice, there is no intervertebral disc located rostral to the vertebral body of the axis^{12,17}, nor in the transgenics was there an intervertebral disc located rostral to the axis (data not shown), although the basioccipital bone has assumed the shape of a vertebral body (compare B and C1-2 in Fig. 3e and f) and has ectopic neural arches (E⁺) fused to it (compare Fig. 2a and c with b, d, e and f; see also B in Fig. 2g and h). Hence, the transgenic basioccipital bone is

assuming vertebral characteristics, in particular, atloaxoid characteristics, although it does not appear to be resegmented. Furthermore, in the transgenics the occipital condyle (CO) is absent, and the exoccipital bone (and probably the supraoccipital bone) appear to have retained their early (somite stage) segmental arrangement dorsolaterally, giving rise to one to four ectopic neural arches (compare in Fig. 4e, normal and transgenic vertebral arch). The respecification of the developmental fate of the occipital bones by *Hox-4.2* is probably mediated by an altered interpretation of axial position cues in the developing occipital somites. The sum of these modifications suggest that a cervical vertebra phenotype, in particular, an atloaxoid (C1-2) phenotype, has been imposed upon the occipital bones. Hence, this transformation is towards a more posterior phenotype, and can be interpreted as a homeotic posteriorization or transformation¹⁸ similar in principal to what has been observed in *Drosophila* by ectopic HOM-C gene expression⁷⁻¹⁰ (reviewed in ref. 3).

The modifications resulting from ectopic *Hox-4.2* expression were limited to paraxial mesoderm-derived structures anterior to the atloaxoid (C1-2) complex. The absence of overt segmentation of the basioccipital in the transgenics suggests that the spatial interactions affecting vertebral segmentation are independent for dorsolateral and ventral components. Furthermore, the character of the transformation in the transgenics (that is, ectopic neural arches) was invariant; but, the number of ectopic neural arches was inconstant, suggesting that segmentation and segmental character are separate developmental issues. Interestingly, within this region, there was no clear alteration of myotome-derived structures (see, for example, Fig. 3i), nor was there any visible transformation or ectopic production of cranial nerves, cranial motor nuclei, or cranial ganglia in the hindbrain, although the *Hox-1.6* promoter is active in this region (Fig. 1b, and data not shown). This possibly reflects a tissue-specific limitation of the *Hox-4.2* protein (that is, its patterning ability may be restricted to sclerotome cells), or it might reflect a more refractory nature of myotome and neuroectoderm to ectopic *Hox* gene expression. *Hox* genes expressed in the same region of the embryo can be responsible for patterning different tissue types as has been shown by the gene disruption studies⁴⁻⁶. Further gene disruption and ectopic expression studies with other *Hox* genes (in particular, the other paralogues of previously tested genes) will be necessary to establish whether all *Hox* genes show a tissue-specific restriction within their domain of activity.

Our results suggest that a 'posterior' *Hox* gene has the ability to initiate the repatterning of anterior skeletal structures. This is likely to be mediated through the activation of many downstream effectors of the *Hox-4.2* gene. Whether other *Hox* genes are among these downstream effectors, or if there is significant 'cross-talk' between ectopic *Hox-4.2* and other *Hox* genes in the transgenic embryos is unknown. It is clear, however, that the paralogous *Hox-1.4* expression domain is not affected in the transgenic embryo (Fig. 1b); hence it is probably not an essential component of the observed homeotic transformation. Transgenic mice bearing the *Hox-1.1* gene under the control of a ubiquitously expressing β -actin promoter displayed structural changes limited to that embryo region anterior to the normal domain of *Hox-1.1* expression^{19,20}. Furthermore, exposure of mouse embryos to retinoic acid, which altered *Hox* gene expression along the antero-posterior axis²¹, resulted in axial skeleton transformations in a manner consistent with the 'posterior prevalence' model.

Interestingly, a number of aspects of the phenotype of the *Hox-1.6* promoter-*Hox-4.2* cDNA transgenics resemble those of phylogenetically more primitive species. The affected region corresponds essentially to the neocranium, which evolutionarily is thought to be a more recent addition to the head^{15,22}. The neocranium is believed to have arisen by assimilation of vertebral elements caudal to the X cranial (vagus) nerve into the skull, whereas the palaeocranium, which encompasses the trabecular

FIG. 3 Histological sections of day-18.5 normal and transgenic newborns. Frontal sections through the heads of a transgenic (a, c, e, g, i, k) and of a normal (b, d, f, h, j, l) newborn, stained with haematoxylin and Mallory's trichrome (transgenic, in blue) or with Mallory's phosphotungstic acid-haematoxylin (normal, in red). Sections a-d show aspects of the cervico-occipital junction at the level of (a, b) or immediately in front of (c, d) the foramen magnum (curved black arrow) through which the spinal cord (S) enters the skull. The absence of the occipital condyle (CO) in the transgenic newborn results in the absence of the joint (atlanto-occipital joint, straight white arrow in b) between this bone and the arch of the first cervical vertebra, the atlas (C1A). This might lead to cervical disjunction, which could be the cause of death in the transgenics. The presence of an ectopic neural arch (E⁺, in c) is indicated. Note that in the normal newborn, the axis dens (D), located at the tip of C1-2, represents the most anterior tip of the vertebral column, and does not make contact with the basioccipital bone (B), which lies anterior to the plane of the section in d. Sections e and f are at the level of the foramen jugular (curved white arrow), which transmits the internal jugular vein and cranial nerves IX, X and XI. The normal basioccipital bone (B, section f) is a flat bone which, at this developmental stage, is no longer in contact with the notocord (NT). g and h, Sections at the level of the basisphenoid canals (curved black arrow) through which the internal carotid arteries (I) enter the skull. i and j, Sections through the face at the level of the first upper and lower molars (MU and ML, respectively). Note that the tongue muscles that are derived from the myotomes of the occipital somites¹⁶ are normal in the transgenic newborn. k and l, Sections showing aspects of the cerebellum and hindbrain. The absence of fusion of the palatal processes might be an indirect consequence of the cervical column abnormalities (ref. 30, and refs therein). Likewise, it should be noted that the bones of the skull are formed by both endochondral and perichondral ossification from a cartilaginous model and that the defects observed in the transgenics could result, in part, from abnormal secondary growth rather than a direct effect of the transgene. The ectopic neural arches observed in the transgenics were not associated with ectopic intervertebral muscles, indicating a limitation of the transformation phenotype to sclerotome-derived structures. In the cases where the supraoccipital and exoccipital bones were absent (Fig. 2b and d), the neck muscle attachment sites to these bones were undetectable. The otic capsules appear normal in both position and size. In addition, the neural crest-derived tympanic rings appear normal. Interestingly, no ectopic sensory ganglia were detected beside the caudal hindbrain in the transgenics, indicating that the transformation phenotype does not extend to neurogenic neural crest-derived tissues (see text for discussion). Magnifications: $\times 34$ in a-f, i and j; $\times 85$ in g, h; $\times 17$ in k, l. Abbreviations: B, basioccipital bone; BS, basisphenoid bone; C, cerebellum; C1-2, fused bodies of the first and second cervical vertebrae; C3, body of the third cervical vertebra; C1A and C2A, neural arches of the first and second cervical vertebrae, respectively; CO, occipital bone condyle; D, axis dens; E, exoccipital bone; E⁺, ectopic neural arch; E⁻, exoccipital bone remnant; H, hypophysis; HB, hindbrain; I, internal carotid artery; L, transverse ligament of the atlas; MB, midbrain; ML and MU, first upper and lower molars, respectively; N, nasal septum; NT, notochord; NP, nasopharyngeal duct; P, pharynx; PP, palatal processes; S, spinal cord; T, cartilaginous otic capsule; TO, tongue; 3V and 4V, third and fourth ventricles, respectively. METHODS. Newborns were delivered by caesarean section and a tissue sample taken for DNA analysis as for Fig. 2. Newborns were skinned and fixed for at least 1 week in Bouin's fixative (Sigma), dehydrated in graded ethanol, embedded in paraffin and sectioned in a frontal plane⁵. Sections were stained with either Groat's haematoxylin and Mallory's trichrome (M.M., unpublished) or with Mallory's phosphotungstic acid-haematoxylin.

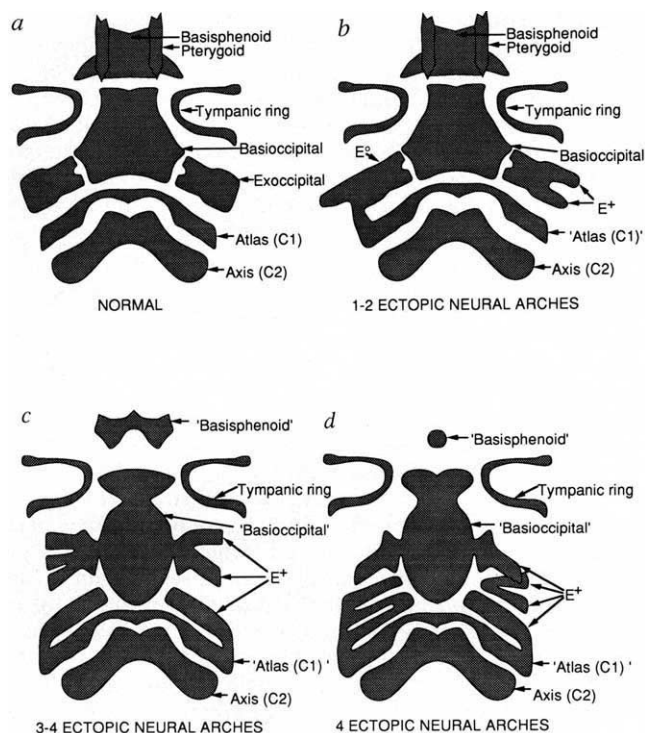
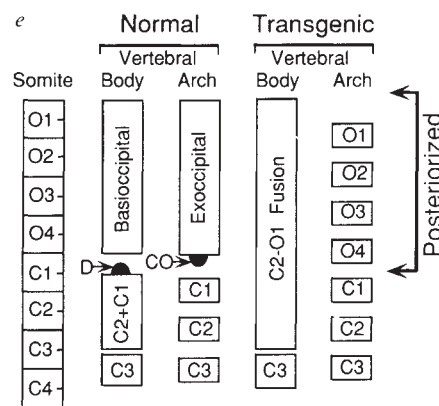


FIG. 4 *a-d*, Schematic representations of the ventral braincases of normal (wild type) and transgenic animals, with lateral elements splayed out to the side. Dorsal elements are not shown. Note the correlation between the number of ectopic neural arches and the anatomy of the basisphenoid and basioccipital bones. In the most extreme cases of transformation in the transgenic animals, which resulted in 3–4 ectopic neural arches (*c, d*), the ectopic neural arches (E^+) appear to predominate, in part, at the expense of the basisphenoid and basioccipital bones. *a*, Wild-type; *b*, 1–2 ectopic neural arches; *c*, 3–4 ectopic neural arches; *d*, 4 ectopic neural arches. Positions of the basisphenoid, pterygoid, tympanic ring, basioccipital, exoccipital, atlas (C1) and axis (C2) are indicated. Names written in quotation marks indicate that the bone no longer retains wild-type anatomy. E^0 , exoccipital bone remnant; E^+ , ectopic neural arch. *e*, Schematized view of



the occipito-cervical region in normal (wild type) (left) and transgenic (right) animals. During normal development of the vertebral column, each prevertebra is formed from the fusion of the cranial half of one sclerotome with the caudal half of the next-rostral sclerotome^{1,3,4}. A vertebra is composed of two major parts: (1) the vertebral body or centrum, which surrounds the notochord, and which is separated from adjacent vertebral bodies by intervertebral discs; and (2) the neural arch, which extends dorsally from the vertebral body to surround the spinal cord^{1,12}. The atlas (C1) is an exception, as it does not possess a vertebral body. Instead, the vertebral body primordia of atlas fuses with that of the axis (C2) to give rise in part to the axis dens (D), which with the corresponding neural arches of the atlas and the axis, and a contribution from the cranial half of the third somite (C3), form the atlantoaxial complex^{15,17,31}. The tip of the axis dens as well as the occipital condyles (CO) are derived from the sclerotome of the cranial half of the first cervical somite (C1)^{15,22}. The occipital condyles (CO) are fused to the exoccipital bone. In normal mice, parts of the exoccipital and basioccipital bones result from the fusion of the sclerotomes of the 4 occipital somites (O1–O4). In the transgenics (right) there is a fusion between the axis dens (D) and the basioccipital bone. In addition, there are from one to four ectopic vertebral arches now present between the interparietal bone (not shown) and the vertebral arch of atlas (C1). Transformed or 'posteriorized' region in the transgenics is indicated. The absence of the supraoccipital bone in the transgenics is not shown.

Abbreviations: CO, occipital condyle; C1–C4, cervical somites, bodies or arches of cervical vertebrae (depending upon the column); D, axis dens; O1–O4, occipital somites, bodies or arches of cervical vertebrae (depending upon the column).

and parachordal cartilages, is the more ancestral portion of the head and lies immediately rostral to the neocranium^{15,22}. Atavistic changes (changes in structure that resemble phylogenetic origin) were observed in the transgenics, and include: (1) the presence of clearly segmented neural arches arising from the most anterior somites (which normally give rise to the occipital bones in higher vertebrates; see Fig. 4 for summary); (2) the hypophysis lying in direct contact with the pharynx. These alterations are similar to characteristics that specifically distinguish the most primitive vertebrates, the *Agnatha*, of which *Petromyzon marinus* (the lamprey) is a member, from higher vertebrates^{17,22}. The *Agnatha* lack a neocranium and instead have the palaeocranium positioned immediately rostral to the vertebral column^{15,22}. Atavistic changes were also observed in the actin-*Hox-1.1* transgenics, but the modifications were different and resulted in mice bearing a 'proatlas'^{1,20}. In no case have we observed an intervertebral disc rostral to the vertebral body of axis in the mice transgenic for the constructs shown in Fig. 1*a*, nor have we observed any supernumerary anterior arches of atlas, which were observed in the retinoic acid-treated embryos²¹. Thus the transgenic animals presented here do not bear a 'proatlas', nor do they display any retinoic acid embryopathy characteristics (apart from a cleft palate), which were observed in the actin-*Hox-1.1* transgenics¹⁹. Hence, different combinations of promoters and *Hox* genes clearly produce different phenotypes in the resulting transgenic animals.

Would ectopic expression of additional *Hox* genes be required to convert fully the neocranium into vertebrae? Our results raise the possibility that *Hox-4.2* is normally expressed in the most rostral somites in *Agnatha*, and that this expression was lost during evolution, concomitant with the origin of an advanced neocranial phenotype^{15,22}. In any case it is interesting that ectopic expression of a single *Hox* gene can result in an extensive reversion towards a phenotype resembling a phylogenetically more primitive vertebrate species. □

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Enterotoxin residues determining T-cell receptor V β binding specificity

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SUPERANTIGENS such as the staphylococcal enterotoxins bind to major histocompatibility complex (MHC) class II molecules and activate T cells through a specific interaction between the V β region of the T-cell antigen receptor (TCR) and the toxin^{1–4}. The TCR β -chain alone is sufficient to produce the interaction with the enterotoxin–class II complex⁵. Identification of the regions of enterotoxins that interact with TCR has so far proved equivocal because of difficulties in distinguishing between direct effects on T-cell recognition and indirect effects resulting from alteration of binding to class II. For example, amino-terminal truncations of SEB abrogated T-cell stimulation whereas carboxy-terminal truncation of SEA stopped its mitogenic activity^{10,11}. The most comprehensive study to date, accounting for both enterotoxin binding to class II and enterotoxin interactions with the TCR, identified two functionally important regions for SEB binding to TCR¹². Although the amino-acid sequences of staphylococcal enterotoxins A and E are 82% identical⁶, they activate T cells bearing different V β elements. We have assayed the binding of cells coated with these enterotoxins to soluble secreted TCR β -chain protein and find that V β 3 binds enterotoxin A but not E, whereas V β 11 binds enterotoxin but not A. To map the amino-acid residues responsible for these different binding specificities, we prepared a series of hybrids between the two staphylococcal enterotoxins. We report that just two amino-acid residues near the carboxy terminus of the enterotoxins are responsible for the discrimination between these molecules by V β 3 and V β 11. The crystal structure of staphylococcal enterotoxin B (ref. 12) indicates that these form part of the outer rim of the site recognizing TCR.

We have exploited the similarity between the staphylococcal enterotoxin A and E (SEA and SEE) sequences (>90% similarity¹, 82% identity⁶), their similar predicted secondary structures¹³, and their different V β specificities to investigate the regions of the enterotoxin molecule contributing to the specific interaction with different V β elements. Both SEA and SEE bind with nanomolar affinity to class II and activate T cells at similar concentrations (Fig. 1), so we were able to analyse the residues

responsible for V β specificity without affecting the ability of the molecule to bind either class II or the TCR. We used purified soluble TCR β -chain¹⁴ in a cell-binding assay to compare the interaction of V β 3 and V β 11 β -chains with SEA and SEE. The V β 3 β -chain bound to SEA but not SEE, whereas the V β 11 protein bound SEE but not SEA (Fig. 2b and c). The results with SEE were predictable from studies of T-cell activation by enterotoxins, but SEA would be expected to bind both V β 3 and V β 11 as it activates both types of T cells^{2,5,6} (Table 1). This discrepancy may be due to a greater specificity in the binding assay compared with T-cell activation *in vitro*, when a number of non-antigen-specific ligand–receptor interactions are also involved. Alternatively, it could be an effect of V α or even DJ β on V β recognition of superantigens^{15,16}.

To delineate the amino-acid sequences responsible for the SEA and SEE binding phenotypes, a series of hybrid molecules between SEA and SEE was made, dividing the molecules roughly into thirds (Fig. 1). A further series of hybrids, concentrating on the sequence differences between SEA and SEE at the carboxy terminus was subsequently made. Hybrid molecules were tested for their ability to bind to MHC class II-bearing cells and to activate T cells. All bound class II molecules with dissociation constants in the nanomolar range and activated T cells at 0.3–5.0 $\mu\text{g ml}^{-1}$. Differences in affinity for class II did not correlate with potency in T-cell activation assays, similar to results comparing wild-type toxins¹⁷.

The binding of cells treated with the hybrid enterotoxins AEE, AAE, EAA and EEA showed that the specificity for V β 3 residues in the C-terminal 76 amino acids of SEA (Fig. 2a). This experiment also illustrates that any binding is independent of the affinity of these hybrids for MHC class II. Both AEE and SEE have greater binding affinity for class II than do EAA or EEA but do not bind to V β 3. The region for V β -recognition specificity was further narrowed using the hybrids C18 (SEA with residues 215–250 from SEE) and D18 (SEE with residues 210–250 from SEA). Reciprocal binding of hybrids D18 and C18 to V β 3 and

TABLE 1 Per cent V β ⁺ cells in the Thy 1.2⁺ population

C57C1/6 response to:	V β 3	V β 6	V β 11
Con A	2.7	7.4	6.3
SEA	41.6	2.1	24.8
SEE	0.7	1.5	72.1
C19	4.6	1.3	64.0
D19	48.1	1.7	31.1

The table shows activation of V β 3 and V β 11 cells by SEA, SEE and hybrid molecules. Flow cytometry was used to analyse the V β -specificity of the C19 and D19 mutant enterotoxins in comparison to wild-type SEA and SEE molecules. In contrast to the cell-binding data, SEA activates V β 11⁺ cells as well as V β 3⁺ cells. Hybrid D19 shows the same specificity. The hybrids EEA, EAA and D18 had the same specificity as SEA. The SEE pattern of stimulation was obtained with AAE, AEE and C18 (data not shown). T-cell activation data may reveal relatively weak interactions between the TCR and the class II–enterotoxin complex or an effect of V α or DJ β . SEE and C19 both activate V β 11⁺ cells but not V β 3⁺ cells. Spleen and lymph node cells from C57BL/6 mice were prepared as single-cell suspensions, washed 3 times and resuspended in RPMI 1640 medium containing 10% FCS, L-glutamine, 2-mercaptoethanol and penicillin streptomycin at a cell density of 5×10^6 cells per ml. Cells were incubated with 0.5 $\mu\text{g ml}^{-1}$ of enterotoxin or 2.5 $\mu\text{g ml}^{-1}$ concanavalin A (ConA) for 3 days at 37 °C. After 3 days, cells were washed and cultured in the presence of interleukin-2 (100 U ml^{-1} for 2 days). Live cells were collected on a Ficoll–Paque (Pharmacia) density gradient, washed in PBS with 0.1% BSA and 0.02% sodium azide at 4 °C. Cells were stained with antibodies to detect V β 11 (RR3-15; ref. 18), V β 3 (KJ25; ref. 19), or V β 6 (RR4-7-FITC; ref. 20) followed by anti-rat-FITC (Jackson) or anti-hamster-FITC (Caltag) to detect RR3-15 or KJ25-labelled cells, respectively. Cells were incubated with 15 μl normal rat serum followed by anti-Thy1.2-PE (Pharmingen). After addition of propidium iodide cells were analysed on a FACScan (Becton Dickinson), gating on propidium iodide-negative (live) cells. Data are presented as the percentage of Thy 1⁺ cells staining with each antibody. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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V β 11, respectively, showed that the specificity-determining region was the stretch of amino acids from 210–250 (Fig. 2b). In this region there are six amino-acid differences (one-letter code) between SEA and SEE, G214D, S220P, N221D, M241L, I245L and S150T (SEA/position/SEE). Because the changes at residues 214, 220 and 221 are clearly non-conservative, we made the chimaeric enterotoxins C19 and D19 (Fig. 1). Mutant C19 is SEA except for residues 220P and 221D which are from SEE. Mutant D19 is SEE except for residues 214G, 220S and 221N which are from the SEA sequence. Figure 2c illustrates that switching these residues between SEA and SEE changed their specificity for binding to a particular V β . Thus V β 3 differentiates between SEA and SEE on the basis of at most three residues, and V β 11 recognizes SEE but not SEA using at most two residue differences between these enterotoxins.

These data are supported by T-cell activation experiments: SEA activates T cells bearing both V β 3 and V β 11 (refs 8, 9 and

Table 1), and the mutant D19 activates T cells with both of these V β regions. Hybrids EEA, EAA and D18 gave the same pattern, and activation of T cells by SEE and C19 was specific for V β 11, not V β 3, as were hybrids AAE, AEE and C18 (Table 1 and data not shown).

Secondary structure predictions place SEA and SEE residues 220 and 221 and SEB residues 63 and 64 on β -turns^{12,13}. The X-ray crystal structure of SEB has now been solved¹⁷. This shows that the N-terminal regions identified by mutagenesis as being important in the TCR interaction¹² (25N, 63N and 64Y) are part of a pocket in the molecule accessible to the TCR. Most important for this discussion is the finding that residues 220F and 221D are on a β -turn and located on the outer rim of this putative TCR-binding site. From the crystal structure, residues 220–226 of SEB may be part of the TCR interaction site¹². This explains an apparent discrepancy between our results and other studies on the regions of enterotoxins interacting with TCR¹². V β 3 and V β 11 distinguish between SEA and SEE using residues 220 and 221. These toxins are invariant at residues 63 and 64, so the possible involvement of these amino acids, which are important in V β 7 and 8 recognition¹² cannot be determined.

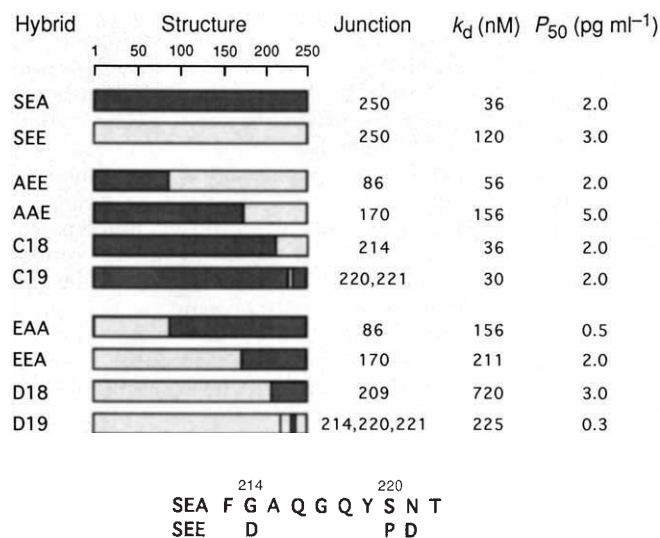
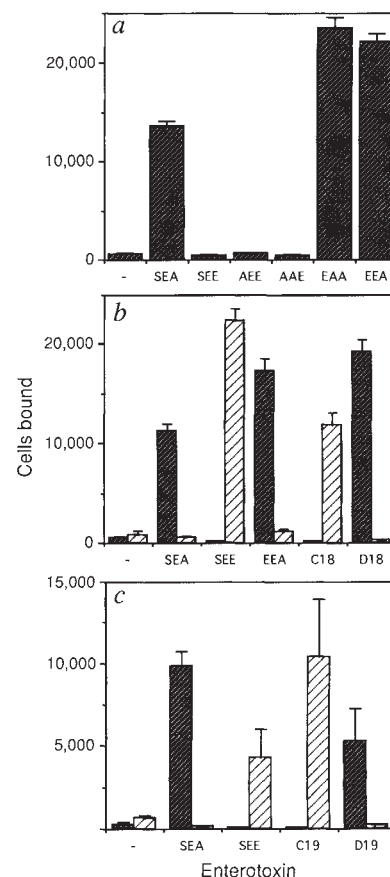


FIG. 1 Schematic representation of the hybrid enterotoxin molecules showing SEA (dark shading) and SEE content (light shading). The boundary between the SEA and SEE sequences in the hybrids is shown. For example, AAE represents residues 1–170 of SEA followed by residues 171–250 of SEE (numbering according to ref. 1). C19 is an SEA molecule, except for amino acids 220 and 221 from SEE. The sequences of SEA and SEE in the region covered by mutants C19 and D19 are presented, blank spaces indicating identity. Also shown is the dissociation constant (K_d) for binding of each toxin molecule to class II and the concentration of each toxin producing half-maximal proliferation of T cells (P_{50}). All the mutant toxins bind to class II with nanomolar affinity and activate T cells at similar concentrations to the parent molecules, suggesting that they are correctly folded. This is supported by the fact that they are all resistant to trypsin digestion (data not shown). The variations in K_d suggest that differences in strength of class II binding between SEA and SEE are the result of sequences spread throughout the molecules.

METHODS. The mutant toxins were constructed by polymerase chain reaction overlap and cloned into pGEX1T (Pharmacia). The fusion protein was induced with IPTG, released from *E. coli* by sonication and purified by affinity chromatography on glutathione-agarose. The active toxin molecule was released from the column by cleavage with bovine thrombin and each was found to be >95% pure by SDS-PAGE. Sequencing revealed additional mutations in certain of the molecules in which the wild-type toxin residue is given first followed by its sequence position and then the mutant residue: AEE (A22D, (G56D, K115R), AAE (V164A), SEE (K115R), EEQ (Y89F), D18 (K115R, H126Q), and D19 (K115R, H126Q). Binding of enterotoxins to class II was determined by competitive inhibition of each toxin of ¹²⁵I-labelled SEA binding to LG-2 cells (HLA-DR1 homozygous) at 37 °C. Standard errors in these experiments were less than $\pm 15\%$. The amount of hybrid enterotoxin required to inhibit 50% binding of ¹²⁵I-SEA was then normalized against the amount of SEA required to reach the same level of inhibition. The K_d was calculated from the K_d for SEA, determined by Scatchard analysis. The ability of each toxin to stimulate T cells was determined in standard proliferation assays using peripheral blood lymphocytes.

FIG. 2 Carboxy terminal residues in SEA and SEE determine recognition and binding by V β 3 and V β 11 TCR β -chains, respectively. Cell binding was assayed with either V β 3 (dark shading) or V β 11 (light shading). a, Binding of V β 3 localizes to the C-terminal 76 amino acids of SEA. b, Specificity of binding of V β 3 and V β 11 to SEA and SEE, respectively. C18 and D18 binding shows that this specificity resides in the C-terminal 33 residues of SEE and the C-terminal 37 residues of SEA. c, The specificity of binding of V β 3 for SEA is determined at most by residues 214, 220 and 221 and the specificity of binding of V β 11 for SEE is determined by residues 220 and 221.

METHODS. Cell binding was assayed as described⁵. Briefly, anti-TCR β -chain monoclonal antibody H57-597 (ref. 21) was coated onto 96-well ELISA plates which were then blocked with FCS (1%), following which soluble β -chain (50 μ l of 20 μ g ml⁻¹ solution) was allowed to bind for 2 h at 37 °C. Wells were washed with PBS three times and 10⁵ [³⁵S] methionine-labelled Raji cells (class II-expressing) coated with the appropriate enterotoxin were added in 100 μ l in PBS-FCS (5%). Plates were incubated 2 h at 37 °C. Wells were washed and specifically bound cells were lysed for counting. The number of cells bound was calculated from the specific activity of the cells. Soluble V β 11 β -chain was prepared using the V β 11, D β 2.1, J β 2.1 exon from the T-cell clone F5 (ref. 22) expressed as part of a truncated β -chain¹⁴. Soluble V β 3 β -chain was prepared from a similar construct expressed in baculovirus. The V β 3, D β 2.1, J β 2.5 gene construct described¹⁴ was cloned into pVL 941 and expressed in baculovirus by standard techniques²³. Secreted β -chain was purified on an H57-597 affinity column⁵. Enterotoxins used here were purchased from Toxin Technology (SEA), or prepared from recombinant *E. coli* (see Fig. 1 legend). Enterotoxins were added to cells (100 μ g ml⁻¹) at the time of metabolic labelling. Labelled cells were washed 3 times in PBS-FCS (1%) and subsequently resuspended at 10⁶ cells per ml in PBS-FCS (5%).



Along with the invariant residue 25N, these amino- and carboxy-terminal regions form an important part of the TCR recognition site of the toxin molecule. □

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Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis

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INEFFICIENT vascular supply and the resultant reduction in tissue oxygen tension often lead to neovascularization in order to satisfy the needs of the tissue¹. Examples include the compensatory development of collateral blood vessels in ischaemic tissues that are otherwise quiescent for angiogenesis and angiogenesis associated with the healing of hypoxic wounds². But the presumptive hypoxia-induced angiogenic factors that mediate this feedback response have not been identified. Here we show that vascular endothelial growth factor (VEGF; also known as vascular permeability factor) probably functions as a hypoxia-inducible angiogenic factor. VEGF messenger RNA levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia and return to background when normal oxygen supply is resumed. *In situ* analysis of tumour specimens undergoing neovascularization show that the production of VEGF is specifically induced in a subset of glioblastoma cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic regions) and the clustering of capillaries alongside VEGF-producing cells.

To investigate a natural situation of imbalanced vascularity, we analysed the angiogenesis-dependent growth of glioblastoma tumours³. Glioblastoma multiforme (gm), a malignant human primary intracranial brain neoplasm, seemed most suitable for this type of analysis because in these rapidly growing tumours the development of the tumour vasculature is often unable to meet the perfusion demands imposed by the rapid expansion of the tumour. The resultant oxygen and nutritional deprivation

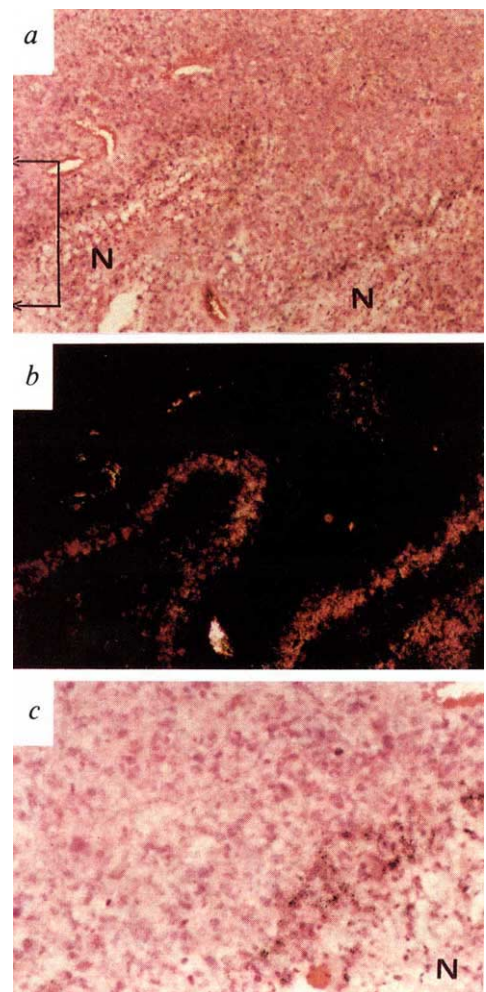


FIG. 1 Specific expression of VEGF mRNA in the periphery of necrotic areas of a glioblastoma tumour. A thin section of gm biopsy hybridized *in situ* with a VEGF-specific probe. *a* and *b*, Bright- and dark-field images, respectively; *c*, enlargement of the area boxed in *a*. Sections were counterstained with haematoxylin and eosin. Note that VEGF-expressing cells are localized alongside the edges of necrotic regions (N).

METHODS. Tumour specimens: Specimens of gm were obtained immediately after surgical removal for histopathological diagnosis. The remainder of the tissue was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline. Fixed specimens were incubated in 0.5M sucrose in phosphate-buffered saline before embedding in Tissue-Tek OCT embedding medium (Miles Scientific) and *in situ* hybridization. Routinely processed formalin-fixed, paraffin-embedded specimens were also taken from the Department of Pathology, Hadassah Hospital, and processed for *in situ* hybridization, which has been described²⁵. Autoradiographic exposure was for 5-9 days. Control hybridizations with a riboprobe in the 'sense' orientation were done for each probe. Hybridization probes were DNA fragments cloned into the polylinker of a PBS vector (Stratagene). These were a 590-bp-long cDNA fragment that included most of the coding region of human VEGF₁₆₅; a 1.8-kb cDNA fragment containing roughly the 3' two-thirds of the coding region as well as the entire 3'untranslated region of mouse VEGF. (The human and mouse VEGF probes detected only the VEGF bands expected in preliminary genomic DNA blotting experiments and gave identical hybridization signals in the *in situ* hybridizations.) Constructs in the PBS vector were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ³⁵S-labelled complementary RNA in either the antisense or sense orientation (using T3 or T7 RNA polymerase, respectively). RNA was fragmented by mild alkaline treatment before use as probes for *in situ* hybridization.

induces extensive necrosis. These changes are accompanied by marked, almost pathognomonic proliferation of endothelial cells, with the formation of vascular glomeruli^{4,5}. We reasoned that a hypoxia-inducible angiogenic factor will be predominantly expressed in a restricted subpopulation of tumour

cells, reflecting the localization of hypoxic microregions. We therefore anticipated that by analysing these tumours *in situ* with respect to the spatial pattern of expression of candidate genes, hypoxia-inducible angiogenic factor might be revealed. VEGF was a particularly attractive candidate as this heparin-binding protein has angiogenic activity in the cornea and in the chorioallantoic membrane^{6,7}; also, the secreted protein is a mitogen with a target-cell specificity restricted to vascular endothelial cells⁸⁻¹⁰.

Biopsy specimens, confirmed as gm on the basis of the usual diagnostic criteria¹¹, were obtained shortly after surgical removal, immediately withdrawn into a fixative, and processed for *in situ* hybridization analysis using VEGF-specific, ³⁵S-labelled antisense ribopobes. *In situ* analysis of mRNA was preferred over *in situ* immunodetection of the encoded protein because the localization of the mRNA unequivocally identifies the producer cells, whereas VEGF might also be secreted and sequestered at some distance from these cells^{8-10,12}. In Figs 1

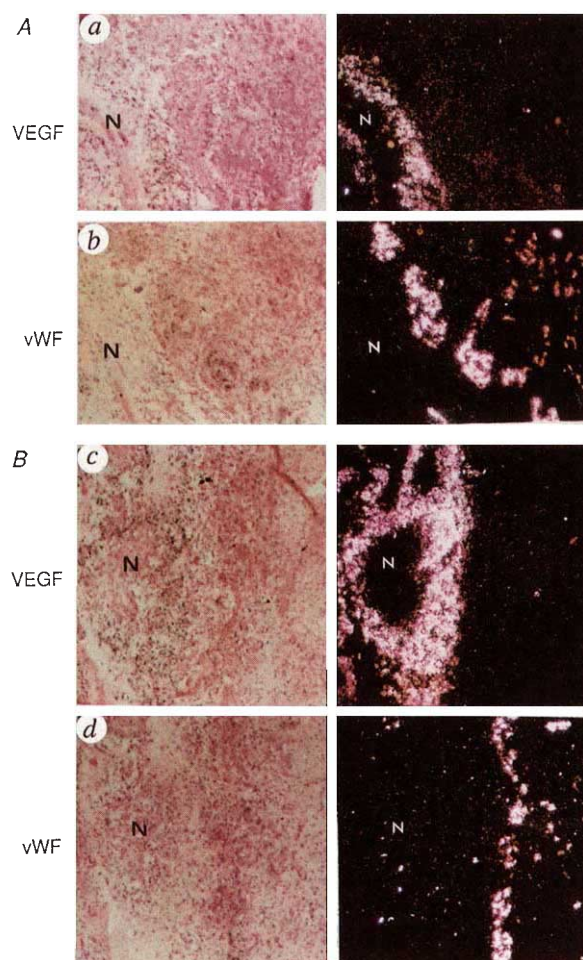


FIG. 2 Clustering of endothelial cells alongside VEGF-producing cells. 10 μ m-thick sections of a gm tumour were hybridized *in situ* with VEGF-specific probe (a, c); adjacent serial sections were hybridized with a vWF-specific probe (b, d; an *EcoRI*-*Sal*I fragment derived from a human von Willebrand factor cDNA clone²⁶). A and B are representative examples of necrotic islets from different regions of the same tumour. The symbol N, depicting the necrotic centre, also serves as a reference point to orient bright- and dark-field images (left and right columns, respectively) and the two adjacent sections. Note that capillary bundles (vWF positive cells) are localized alongside VEGF-producing cells.

and 2, mRNA is seen to be mainly produced in a small fraction of tumour cells, arranged in a stripe-like pattern alongside the periphery of necrotic regions. The highest mRNA levels were detected in tumour cells juxtaposed to cells undergoing necrosis, that is, in cells that presumably experience the most severe hypoxia.

To identify the molecular species of VEGF produced in the tumour, we extracted RNA from gm biopsies from three patients, reverse-transcribed the RNA and amplified the complementary DNA by polymerase chain reaction, using oligonucleotides derived from external exons shared by all differentially spliced VEGF mRNA species (oligonucleotides corresponding to amino acids 92-98 and to the six carboxy-terminal amino acids, respectively)¹³. The principal amplified species detected was a 225-

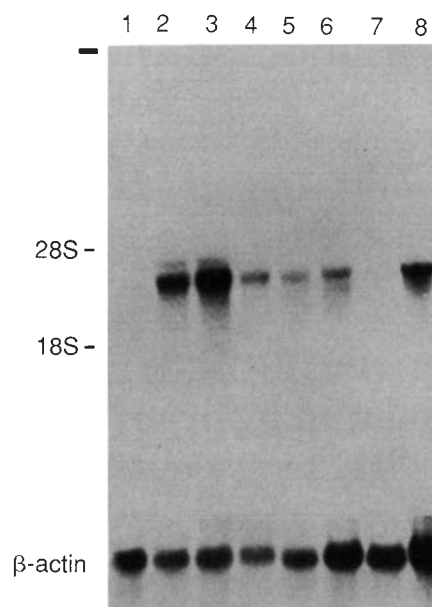


FIG. 3 VEGF expression in cell cultures is hypoxia-inducible. Cultured cells were exposed to different oxygen tensions for 18 h. The cells were collected and their RNA extracted, electrophoresed and blot-hybridized with a VEGF-specific probe. Lanes: 1, C6 cells, normal oxygen; 2, C6 cells, 95% nitrogen; 3, C6 cells, catalyst-induced anoxia; 4, same as in lane 3, followed by 8 h incubation under normal oxygen; 5, C6 cells, 20 h growth under normal oxygen in the presence of 10 μ g ml⁻¹ cycloheximide; 6, C6 cells, grown in the presence of 10 μ g ml⁻¹ cycloheximide, 2 h in normal oxygen and 18 h in catalyst-induced anoxia; 7, L8 cells, normal oxygen; 8, L8 cells, catalyst-induced anoxia. Migration of ribosomal RNA markers (28S and 18S) is indicated.

METHODS. Cell growth under hypoxic conditions: Cell lines used were: C6, a clonal glial cell line derived from a rat glial tumour²⁷ and L8, a myogenic cell line isolated from primary rat skeletal muscle²⁸. C6 cells were grown in Dulbecco-modified Eagle's medium supplemented with 5% FCS. L8 cells were grown in Waymouth medium supplemented with 10% FCS. Two sets of conditions were used to produce an anaerobic atmosphere: (1) cultures of nearly confluent cells were exposed to an atmosphere of 95% nitrogen and 5% CO₂; (2) cultures were incubated in GasPak Plus anaerobic culture chamber (BBL Microbiology Systems) using hydrogen and a palladium catalyst to remove all traces of oxygen. Exposure to hypoxic conditions lasted for 18 h. Isolation and blot analysis of RNA: Total RNA was prepared by the guanidine thiocyanate extraction method, and was purified by centrifugation through CsCl solution²⁹. RNA was denatured in glyoxal and electrophoresed through a 1.0% agarose gel, then transferred onto a nylon-based membrane (GeneScreen Plus, NEN) by capillary blotting and hybridized with the indicated probes. cDNA fragments were labelled with ³²P by randomly primed DNA synthesis. For standardization, filters were rehybridized with a β -actin-specific probe³⁰; the induction index was calculated from densitometric tracings of the autoradiogram, corrected for loading differences as determined by rehybridization with a β -actin-specific probe.

base-pair fragment corresponding to the mRNA encoding VEGF₁₆₅ (data not shown): VEGF₁₆₅ is a secreted form of the growth factor and stimulates proliferation of endothelial cells¹⁴. The potential targets of VEGF is the population of nearby cells expressing VEGF receptors. VEGF receptors are widely distributed on all vascular endothelial cells of adult rat tissues, including quiescent endothelium, but are not found on non-endothelial cells^{14,15}. To visualize blood capillaries better we hybridized adjacent serial sections with a von Willebrand factor (VWF)-specific probe which exclusively identifies endothelial cells irrespective of their proliferation status. In Fig. 2, capillary bundles are seen to be preferentially clustered alongside the stripes of VEGF-expressing cells. Immunohistochemical labelling has shown that VEGF, which is produced elsewhere in the tumour, is concentrated in tumour blood vessels¹². It is therefore tempting to speculate that the proximity of a high concentration of capillaries to sites of VEGF production is the result of a local angiogenic response elicited by the angiogenic factor.

The marked differences in steady-state levels of VEGF mRNA among otherwise indistinguishable tumour cells (Figs 1, 2) can be correlated with their proximity to necrotic centres, where oxygen supply is minimal. We have interpreted this observation to mean that VEGF could be specifically induced in response to hypoxia. It is equally possible, however, that VEGF is induced by factor(s) released from necrotic cells. To distinguish between these two possibilities, we determined whether VEGF expression is regulated by oxygen. Glioma cells were grown under normoxic and hypoxic conditions, and levels of VEGF mRNA were subsequently measured by RNA-blot analysis. Steady-state levels of VEGF mRNA were significantly increased within 18 h of growth under low oxygen tensions (Fig. 3). A 13-fold induction was measured under complete anoxia (as achieved by oxidation with a palladium catalyst). The increase in VEGF mRNA levels was reversible. Upon re-exposure of cells to normal oxygen tension, VEGF expression resumed its low constitutive level (Fig. 3). No evidence for cell death could be detected in cultures exposed to anoxia, and cells continued to proliferate at a normal rate following re-exposure to normal oxygen tension.

These findings support the thesis that the induction of VEGF in gm tumours occurs in response to hypoxia. They also indicate that the rate of release of angiogenic factors by tumour cells might in general be variable, being constantly adjusted according to the changes in the cell microenvironment.

To determine whether VEGF is hypoxia-inducible in other cell types, we did a similar experiment with a cell line of skeletal muscle myoblasts (L8). As shown in Fig. 3, VEGF was also hypoxia-inducible in these cells (5-fold induction after standardization for RNA loads). Similar levels of induction were also observed in a fibroblast (mouse L-cells) line and in primary cultures of cells derived from rat heart muscle (data not shown). We conclude that VEGF is hypoxia-inducible in a variety of cell types. These results suggest that, in principle, VEGF might play a part in ischaemia-induced collateralization responses taking place in normal muscle and connective tissues.

It has been proposed that basic fibroblast growth factor (bFGF) is released by tumour cells, ischaemic myocardium and other tissues in which ischaemia has caused extensive cell damage¹⁶. We could not detect any significant increase in steady-state levels of bFGF mRNA at sites where expression of VEGF was upregulated (data not shown). This finding does not exclude, however, the possibility that cell damage causes the release of pre-made bFGF from its stores¹⁷.

The mechanism(s) that control responsiveness of VEGF to hypoxia are unknown. Precedents for other hypoxia-inducible genes include examples of both transcriptional activation (such as platelet-derived growth factor B-chain gene¹⁸) and post-transcriptional regulation by a *trans*-acting protein that physically interacts with the gene (erythropoietin for example¹⁹). Preliminary results with a general inhibitor of protein synthesis have indicated that VEGF induction depends on prior protein

synthesis (Fig. 3). It also remains to be determined whether increased mRNA production is due to low oxygen, or is secondary to increased lactate in the environment (as in the case of angiogenesis mediated by macrophages recruited to hypoxic wounds²⁰) or to accumulation of other metabolites.

Finally, VEGF has potent vascular permeabilization activity^{21,22}, and hypoxaemia is associated with increased vascular permeability^{23,24}. It is possible that VEGF functions as the link connecting hypoxia and increased vascular permeability. The apparent leakiness of blood vessels in gm tumours⁴ could also be accounted for by the large amounts of VEGF secreted by the tumour cells. □

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Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*

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CLINICAL and experimental studies suggest that angiogenesis is a prerequisite for solid tumour growth^{1,2}. Several growth factors with mitogenic or chemotactic activity for endothelial cells *in vitro* have been described, but it is not known whether these mediate tumour vascularization *in vivo*^{3,4}. Glioblastoma, the most common and most malignant brain tumour in humans, is distinguished from astrocytoma by the presence of necroses and vascular proliferations^{5,6}. Here we show that expression of an endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), is

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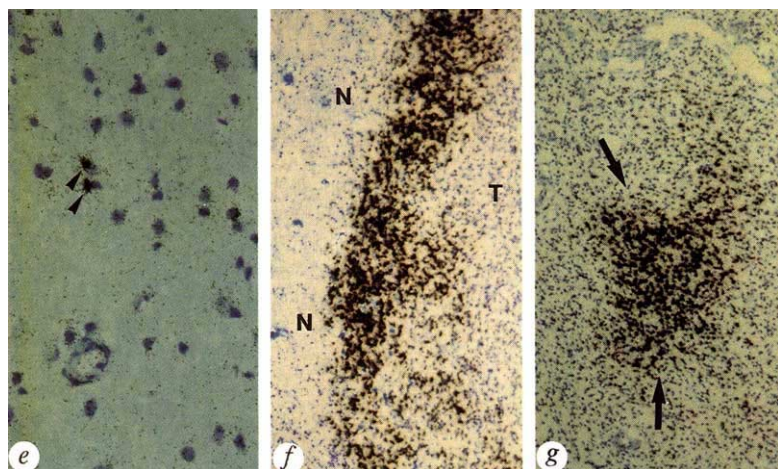


FIG. 1 VEGF mRNA is expressed in some astrocytoma cells but is significantly upregulated in a subset of glioblastoma cells *in vivo*. Frozen sections from low-grade and high-grade glioma hybridized *in situ* with VEGF cRNA. *a-d* (facing page), Low-power microphotographs from low-grade glioma (astrocytoma WHO grade II; *a-b*) and high-grade glioma (glioblastoma multiforme WHO grade IV; *c, d*) show that only a few astrocytoma cells, which cannot be seen at the magnification in *a*, express VEGF mRNA. In contrast, in glioblastoma, VEGF expression is dramatically upregulated in tumour foci (arrowheads in *c*). The tumour cell focus marked with an arrow is shown at higher magnification in *g*. *a* and *c*, Hybridization with antisense RNA; *b* and *d*, control hybridization with the sense probe. *e-g* (left), High-power magnification of astrocytoma (*e*), and two different glioblastomas (*f, g*). In astrocytoma (*e*), VEGF mRNA is expressed in a small number of tumour cells

FIG. 2 VEGF-receptor mRNA is expressed in tumour endothelial cells but not in normal brain endothelial cells *in vivo*. *In situ* hybridization with a VEGF-receptor (*flt*) cRNA on normal brain and glioblastoma. VEGFR mRNA is not expressed in the cortex and white matter from normal adult brain (we did not systematically examine human brain and can therefore not comment on VEGF expression in other regions of the brain) (*a, c*). In glioblastoma, VEGFR mRNA expression is confined to tumour vascular endothelial cells; tumour cells show no hybridization (*b, d*). *a-b*, Bright-field and *c-d*, darkfield images, respectively; L, vessel lumen.

METHODS. Serial sections from the same specimens described in Fig. 1 legend were used for *in situ* hybridization. A 1,080-bp fragment of the human *flt* gene (corresponding to nucleotides 3,233–4,313; ref. 31) was amplified by polymerase chain reaction from human placenta cDNA, cloned into pBluescript vector (Stratagene) and used to generate antisense RNA probe. Exposure time was 10 days. No specific hybridization was observed with the control sense RNA probe.

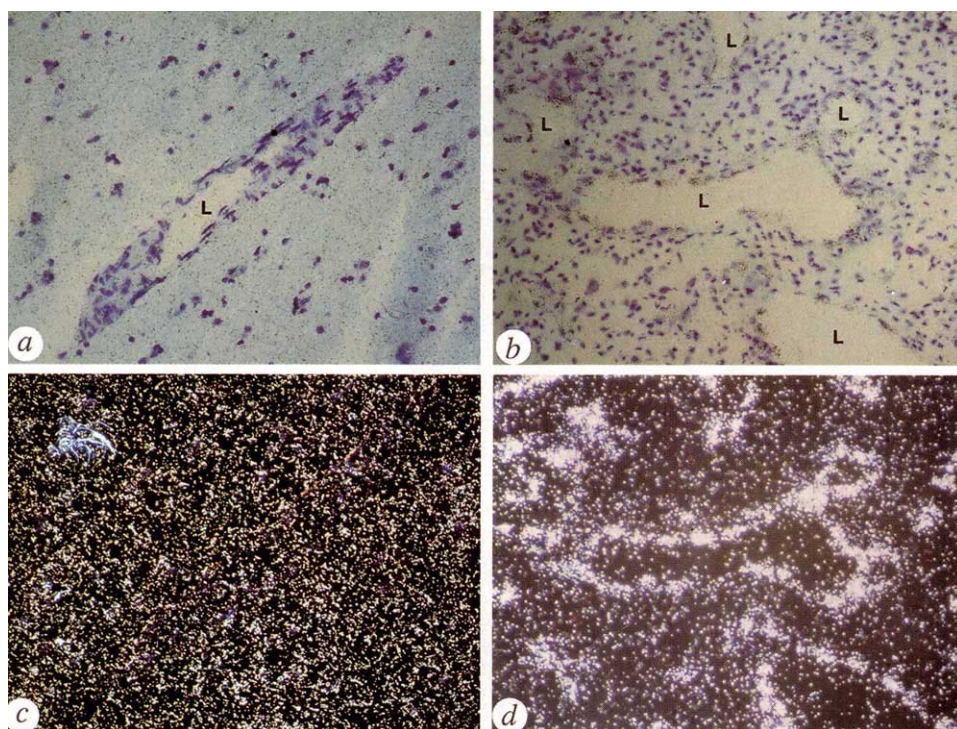
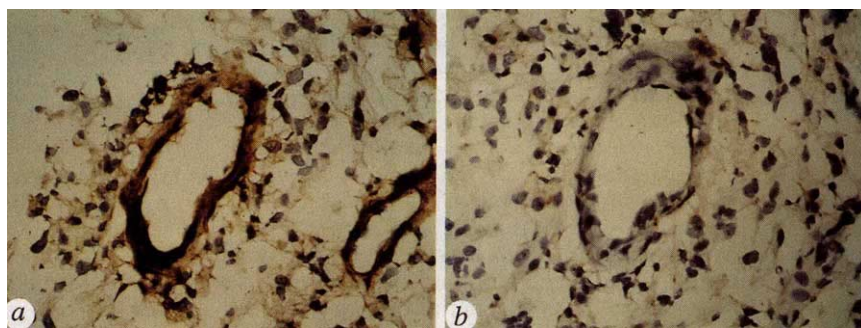


FIG. 3 VEGF protein is present on the tumour vasculature *in vivo*. *a*, Immunocytochemical staining for VEGF shows strong immunoreactivity in the vasculature of a glioblastoma. Glioma cells show only faint immunoreactivity, undistinguishable from background staining. *b*, The immunostaining could be totally abolished by preincubation of the antibody with the peptide. Immunostaining for VEGF is significantly lower in astrocytoma and undetectable in normal brain vasculature (not shown).

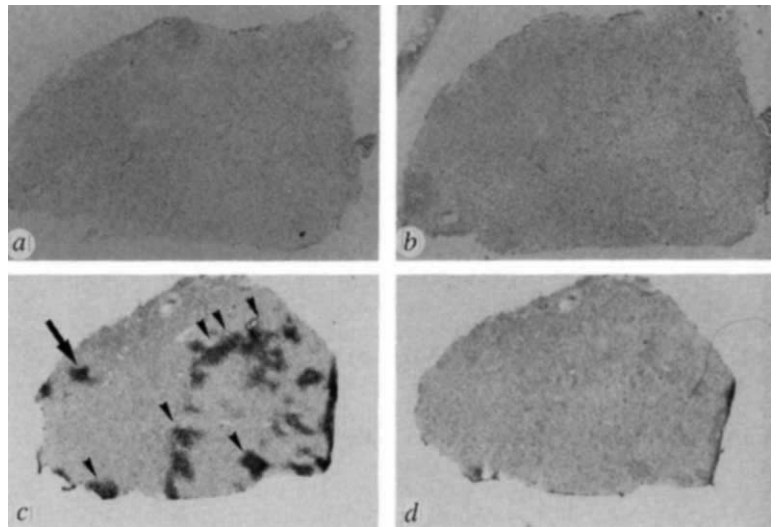
METHODS. Frozen sections were fixed in acetone for 7 min at -20°C . An antipeptide antibody directed against the amino-terminal 20 amino acids of human VEGF (sequence APMAEGGQNHHEV-VKFMVDV) was raised in rabbits and affinity-purified on nitrocellulose coated with $80\text{ }\mu\text{g}$ immobilized human recombinant VEGF₁₆₅. The purified antibody was applied on sections at a concentration of $5\text{ }\mu\text{g ml}^{-1}$. Immunohistochemistry was carried out with a streptavidin-



biotin method as described¹². For control purposes, the antibody was incubated with $100\text{ }\mu\text{g ml}^{-1}$ peptide for 15 min at room temperature.

► (indicated by arrowheads). In glioblastomas, clusters of glioma cells with abundant VEGF mRNA expression were observed. VEGF upregulation was seen in palisading cells that reside along necroses (f) and in groups of glioblastoma cells that were not localized around obvious necroses (arrows in g). N, necrosis; T, tumour.

METHODS. Fresh tumour specimens (two astrocytomas, WHO grade II; two anaplastic astrocytomas, WHO grade III; and four glioblastomas, WHO grade IV) and one normal brain specimen (frontal cortex plus underlying white matter from a 33-year-old female who died of non-neurological disease, obtained 13 h post-mortem) were embedded in Tissue-Tek and frozen in liquid nitrogen. Frozen sections were hybridized *in situ* as described¹⁵. A cDNA clone encoding VEGF₁₆₅ (ref. 18) was used to generate an antisense cRNA probe. Exposure time was 14 days. Sense-RNA probe was used as a control and showed no specific hybridization.



induced in astrocytoma cells but is dramatically upregulated in two apparently different subsets of glioblastoma cells. The high-affinity tyrosine kinase receptor for VEGF, *flt*, although not expressed in normal brain endothelium, is upregulated in tumour endothelial cells *in vivo*. These observations strongly support the concept that tumour angiogenesis is regulated by paracrine mechanisms and identify VEGF as a potential tumour angiogenesis factor *in vivo*.

Angiogenesis, the sprouting of capillaries from pre-existing blood vessels, occurs during embryonic development and certain physiological conditions such as menstruation^{4,7,8}. In addition, endothelial cells proliferate under pathological conditions such as wound healing, eye disease and arthropathy^{7,8}. Angiogenesis is also a prerequisite of solid tumour growth^{2,9}. It has been suggested that the onset of angiogenesis is an early event in tumorigenesis^{9,10} and may facilitate tumour progression and metastasis^{1,11}. It is unknown, however, how angiogenesis is regulated *in vivo*⁷.

Tumour angiogenesis may be regulated by growth factors that are secreted by tumour cells^{2,8}. Several endothelial growth factors with angiogenic activity have been described (reviewed in refs 3 and 4). These include fibroblast growth factors, platelet-derived growth factor (PDGF)^{12,13} and vascular endothelial growth factor (VEGF). VEGF is a dimeric glycoprotein with structural homology to PDGF. Several variants of VEGF have been identified which arise by alternative splicing of messenger RNA¹⁴. We considered VEGF to be a candidate for a tumour angiogenesis factor *in vivo* because (1) the expression pattern of VEGF during mouse development is consistent with a role in embryonic angiogenesis¹⁵; (2) VEGF mRNA is found in some primary tumours¹⁶; (3) it is produced by tumour cell lines *in vitro*^{17,18}; (4) it is a secreted mitogen^{15,17,18}; and (5) its mitogenic activity is apparently restricted to endothelial cells^{19,20}.

In humans, gliomas account for over 60% of primary intracranial neoplasms⁵. Gliomas most commonly arise by malignant transformation of astrocytes and are classified according to histopathological criteria as astrocytoma (low-grade glioma) or glioblastoma (high-grade glioma). Glioblastoma is defined as an astrocytic tumour with (1) high cell density, (2) cellular polymorphism, (3) mitoses, (4) necroses with palisading cells, and (5) prominent vascularization with endothelial cell proliferation^{5,21}. The distinction between low-grade and high-grade glioma has severe implications for the treatment and prognosis of patients. Patients with low-grade glioma have a mean survival time of several years, and are treated by tumour removal. High-grade glioma patients have a mean survival time of less than one year and are treated by tumour removal, post-operative radiation and, occasionally, additional chemotherapy.

Glioblastoma may arise *de novo*, but also forms by malignant progression from astrocytoma. During this progression a remarkable alteration of the vascular phenotype occurs, consisting of prominent endothelial cell proliferation and an increased vessel density⁵. The presence of necroses and endothelial cell proliferation are the most important histopathological criteria for distinguishing high-grade from low-grade glioma^{6,21}. Inhibition of angiogenesis may therefore be an approach to destroying tumours that are highly vascularized and not treatable by conventional methods^{22,23}. Glioblastoma seems to be the prototype of a tumour suitable for anti-angiogenic therapy²⁴.

To investigate whether VEGF is involved in glioma angiogenesis, we hybridized normal brain and glioma tissue *in situ* with ³⁵S-labelled antisense RNA (Fig. 1). VEGF mRNA is not present in the white matter of normal human brain (where gliomas normally arise), but is expressed in cells scattered in the cortex. In low-grade gliomas, VEGF is expressed in few tumour cells. In glioblastoma, we observed a dramatic upregulation of VEGF mRNA expression in two apparently different subsets of tumour cells. As is shown in Fig. 1, VEGF mRNA is strongly expressed in small anaplastic glioma cells which line up in close proximity to necrotic areas. These cells are known as palisading cells⁵. A different pattern of VEGF expression was observed in a glioblastoma, where VEGF is expressed in clusters of tumour cells.

Recently a member of the PDGF receptor family, *flt*, was identified as a high-affinity receptor for VEGF²⁵. By northern analysis, a 7.0-kilobase (kb) *flt* mRNA transcript was found in comparable amounts in astrocytoma and glioblastoma (H.A.W. *et al.*, unpublished results). We performed *in situ* hybridization to investigate the cellular localization of *flt*. As is shown in Fig. 2b, VEGF receptor (*flt*) mRNA is strongly expressed in vascular cells in gliomas and the expression appears to be confined to endothelial cells. The amount of VEGF receptor mRNA seems to be similar in tumour endothelial cells in astrocytoma and glioblastoma, but we could not detect VEGF receptor mRNA in endothelial cells in normal brain (Fig. 2a). This finding suggests that expression of the VEGF receptor is induced in endothelial cells during tumour development.

To investigate whether VEGF protein reaches its target cells, we incubated tissue sections with an affinity-purified anti-peptide antibody directed against human VEGF. Figure 3 shows the strong immunostaining of tumour vascular endothelial cells in a glioblastoma. This finding is consistent with studies by Dvorak *et al.*²⁶, who showed, using a similar antibody, that the protein is present in the vasculature of guinea-pig line-10 tumours. Surprisingly, we found no immunoreactivity in glioblastoma cells. Indeed, given the strong expression of VEGF in

a subset of cells, one would expect to be able to detect VEGF immunocytochemically in those cells. Reasons for the failure to detect VEGF in producer cells may include (1) rapid secretion and diffusion of VEGF from producer cells; (2) failure of the antibody or the detection method to respond to small amounts of VEGF; and (3) accumulation of VEGF in blood vessels by binding to its receptor or by binding to heparan sulphate²⁶.

Two VEGF mRNA transcripts of 3.9 kb and 4.3 kb were found by northern analysis in glioma tissue. Both transcripts were increased up to 50-fold in glioblastoma compared with astrocytoma (H.A.W. *et al.*, unpublished results). Our *in situ* hybridization data confirm these analyses and show in addition that VEGF mRNA is selectively upregulated in a subset of malignant glioma cells during the transition of astrocytoma to glioblastoma. In particular, we observed localized VEGF mRNA upregulation in (1) palisading cells adjacent to necrotic areas and (2) in clusters of tumour cells without obvious adjacent necrosis. This localized upregulation of VEGF expression may be explained in two different ways. First VEGF expression may be inducible by hypoxia. The rapid cell proliferation in the centre of a tumour can lead to increased interstitial pressure, which may lead to compression closure of capillaries and consecutive tissue necrosis²⁷. On the way to necrosis the increasing hypoxia may upregulate VEGF. Alternatively, upregulation of VEGF could be attributed to factors released from necrotic tumour cells. We observed only moderate levels of VEGF mRNA in a glioblastoma with radiation-induced (non-hypoxic) necroses (data not shown). This observation is consistent with the hypothesis that VEGF expression is hypoxia-inducible. Secondly, VEGF-expressing tumour cells could have a growth advantage and proliferate more rapidly than cells that do not express VEGF. Although the nature of palisading cells is not understood, it has been suggested on the basis of histopathological observations that these cells represent a subset of dedifferentiated anaplastic glioblastoma cells⁵. This observation, and our finding of selective VEGF upregulation without adjacent necrosis, is consistent with the hypothesis that VEGF-expressing cells may have a growth advantage. The clusters of VEGF-producing cells could thus represent an early glioblastoma state, which may finally lead to VEGF-producing cells surrounding a necrosis. It has been shown that progression of astrocytoma to glioblastoma involves a clonal expansion of tumour cells carrying mutations in the p53 tumour-suppressor gene²⁸. As p53 mutations can inhibit gene expression in HCT116 human colon carcinoma cells *in vitro*²⁹, it will be interesting to investigate whether mutations in the p53 gene are associated with upregulation of VEGF in the same glioblastoma cells.

Our findings show that the expression of VEGF, an endothelial cell-specific mitogen, is induced in a low proportion of cells in low-grade glioma but is dramatically upregulated in a subset of anaplastic glioma cells. The fact that palisading cells express large amounts of VEGF may explain why endothelial cell proliferation in glioblastoma is particularly apparent in the vicinity of necroses^{5,30}. In addition, we found that the expression of the high-affinity receptor for VEGF, *flt*, is induced in tumour vascular endothelial cells. These findings strongly support the concept that tumour angiogenesis is regulated by a paracrine mechanism, namely the expression and secretion of VEGF by glioma cells and its action on tumour vascular endothelial cells that express the VEGF receptor. Our results identify VEGF as a potential tumour angiogenesis factor in human gliomas *in vivo*. Direct proof for a role of VEGF in tumour angiogenesis will require the inhibition of VEGF-receptor activation *in vivo*. □

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Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets

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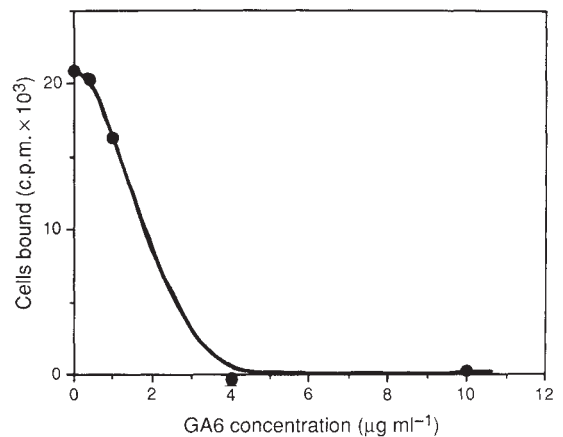
THE glycoprotein P-selectin is a cell adhesion molecule of stimulated platelets and endothelial cells, which mediates the interaction of these cells with neutrophils and monocytes^{1,2}. It is a membrane component of cell storage granules^{3–6}, and is a member of the selectin family which includes E-selectin and L-selectin^{7,8}. P-selectin recognizes both lineage-specific carbohydrate ligands on monocytes and neutrophils, including the Lewis x antigen, sialic acid, and a protein component^{9–12}. In inflammation and thrombosis, P-selectin may mediate the interaction of leukocytes with platelets bound in the region of tissue injury and with stimulated endothelium^{1,2}. To evaluate the role of P-selectin in platelet–leukocyte adhesion *in vivo*, the accumulation of leukocytes within an experimental thrombus was explored in an arteriovenous shunt model in baboons¹³. A Dacron graft implanted within an arteriovenous shunt is thrombogenic, accumulating platelets and fibrin within its lumen. These bound platelets express P-selectin¹⁴. Here we show that antibody inhibition of leukocyte binding to P-selectin expressed on platelets immobilized on the graft blocks leukocyte accumulation and inhibits the deposition of fibrin within the thrombus. These results indicate that P-selectin is an important adhesion molecule on platelets, mediating platelet–leukocyte

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FIG. 1 The effect of monoclonal anti-P-selectin antibody GA6 on the binding of baboon platelets to HL60 cells *in vitro*.

METHODS. BALB/cJ mice were immunized monthly for 3 months with 12 μg of P-selectin emulsified in Freund's adjuvant, and then boosted with P-selectin in saline 4 days before fusion. Hybridomas were prepared by standard methods using P3XAg8.653 myeloma cells²¹. Hybridoma supernatants were screened for binding to P-selectin-coated plates using an ELISA and for inhibition of adhesion of HL60 cell binding to P-selectin-coated plates. Adhesion of HL60 cells to P-selectin coated plates was performed as previously described for E-selectin²². P-selectin (0.25 μg per well in 100 μl of 15 mM NaHCO_3 , 35 mM Na_2CO_3 , pH 9.2) was incubated overnight at 4 °C in 96-well plates. The plates were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 23 °C, washed with RPMI medium in 10% fetal calf serum, before adhesion of [³H]-labelled HL60 cells. In experiments involving antibody, the monoclonal antibody was preincubated on P-selectin-coated plates for 30 min at 23 °C, washed and assays done. Cells producing inhibitory antibodies were cloned by limiting dilution. The clone GA6 was used for these studies. Baboon platelets were obtained from baboon platelet-rich plasma by gel-filtration and immobilized on plates in their activated form. GA6 and AC1.2 (ref. 1), a non-inhibitory P-selectin antibody, were purified from ascitic fluid by protein A chromatography, Fast S ion-exchange chromatography and gel filtration on Sephacryl S200. The gel filtration column was prewashed in 1 M NaOH to remove endotoxin,



and pre-equilibrated with sterile endotoxin-free physiological saline. GA6 and AC1.2 Fab₂ fragments were generated by pepsin cleavage²³ and purified by gel filtration on Superose 6. All batches of GA6 and AC1.2 used had endotoxin levels of less than 1 U per mg protein.

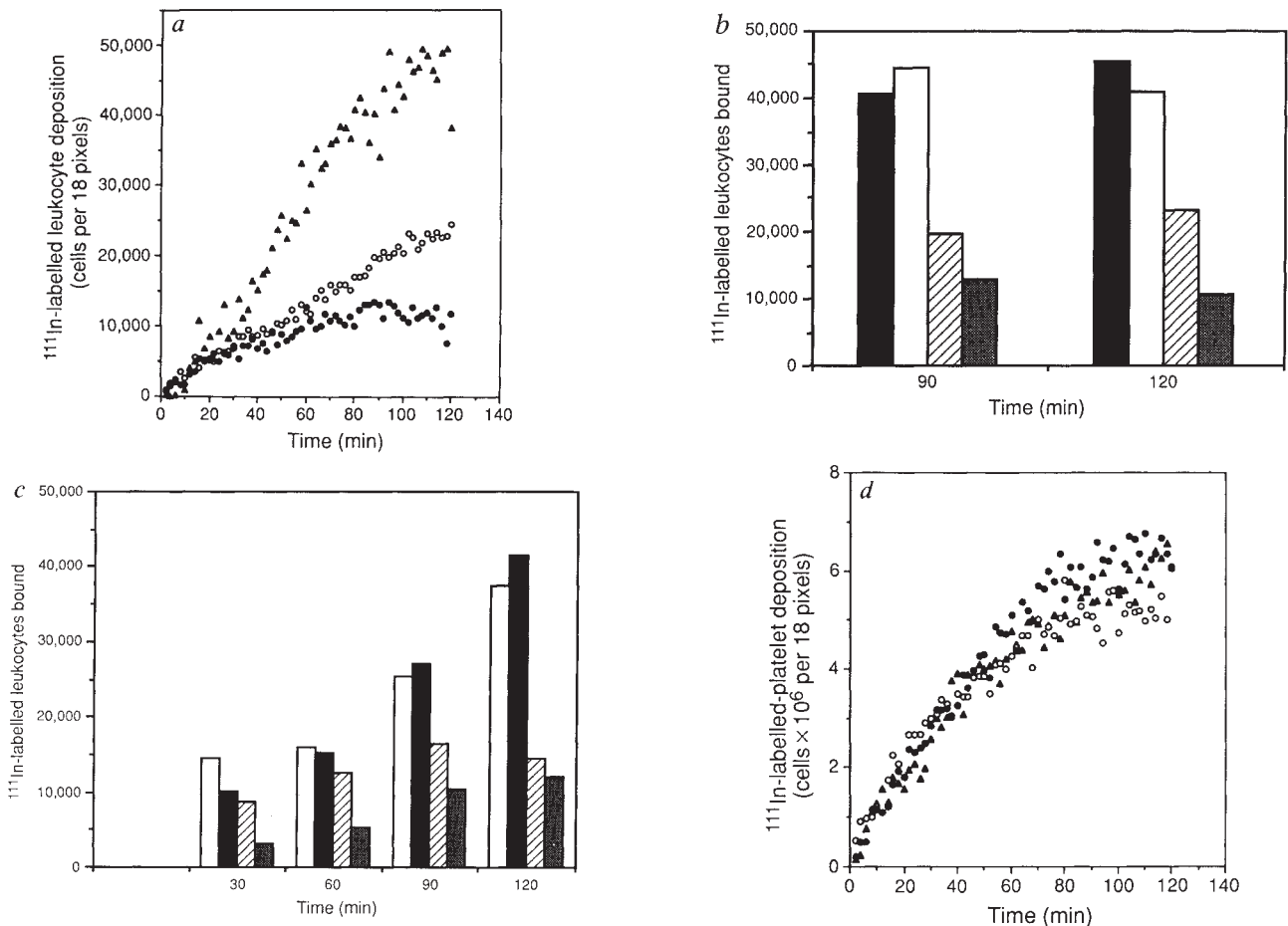


FIG. 2 Inhibition of leukocyte binding to thrombi *in vivo* by an anti-P-selectin monoclonal antibody. *a*, Baboon A. GA6 (○), GA6 Fab₂ fragment (●), no antibody (▲). *b*, Baboon A. GA6 (striped), AC1.2 (white), GA6 Fab₂ fragment (gray) or no antibody (black). *c*, Baboon B. Leukocyte binding is indicated at 30, 60, 90, and 120 min after initiation of flow through the shunt. GA6 (striped), AC1.2 (white), GA6 Fab₂ fragment (gray) or no antibody (black). *d*, Effect of anti-P-selectin GA6 antibody on platelet uptake on the Dacron graft. GA6 antibody infusion (○), GA6 Fab₂ fragment infusion (●), no antibody (▲).

METHODS. Leukocytes were isolated and labelled with ¹¹¹indium^{24,25} by incubation with indium-111 oxine, and the labelled cells washed in citrated plasma and resuspended in 0.9% NaCl. Platelet isolation and labelling were as described²⁶. Leukocytes or platelets were infused 1 h before the introduction of the graft and shunt, respectively. Imaging experiments were per-

formed in baboons using an external Ticoflex (DuPont) shunt between the femoral artery and vein. A 6.0 × 0.5 cm Dacron graft was preclotted by the Sauvage technique and placed in the shunt. Flow through the shunt was maintained at 100 ml min⁻¹. Anti-P-selectin antibody GA6 or AC1.2 or their Fab₂ fragments were filtered through a 0.22 μm membrane filter and injected through the arterial port in the shunt. Serial anterior images were obtained at 2 min intervals for 1 h using an Ohio Nuclear Series 100 Gamma camera. A low-energy, high-resolution, parallel-hole collimator was used with 20% windows over both ¹¹¹In photopeaks. The kinetics of tracer uptake by the graft and blood pool were derived from the radionuclide images by computer analysis, correcting for background and blood-pool activity. Time 0 was defined as the initiation of flow through the shunt. Either GA6, Fab₂ fragments of GA6, Fab₂ fragments of AC1.2 (1 mg per kg body mass of each) or saline was infused into baboon A or B.

binding *in vivo*, that the presence of leukocytes in thrombi is mediated by P-selectin, and that these leukocytes promote fibrin deposition.

Murine monoclonal antibodies were prepared against purified human P-selectin. Hybridomas were screened for anti-P-selectin antibodies that bound to P-selectin and to activated human platelets, and which inhibited the interaction of human platelets and HL60 cells *in vitro*. Of clones GA6, GE12 and AC11, GA6 was employed for the *in vivo* studies because it binds activated baboon platelets and blocks their interaction with HL60 cells (Fig. 1). This antibody does not bind to HL60 cells, neutrophils or lymphocytes, and does not crossreact with L-selectin or E-selectin.

Leukocytes isolated from baboon blood¹⁵ included about 70% neutrophils, 20–30% monocytes, and less than 10% lymphocytes. ¹¹¹In-labelled leukocytes (425 μ Ci; $1-10 \times 10^7$ cells) were infused into a baboon. One hour later, a Dacron graft preclotted in whole blood¹⁶ was introduced into an arteriovenous fistula between the femoral artery and vein. The kinetics of ¹¹¹In-labelled-leukocyte uptake by the graft were derived from serial images produced with a gamma camera. Leukocytes accumulated in the shunt for 2 h (Fig. 2a), but no change in blood leukocyte concentration was observed. To determine whether leukocyte deposition in the thrombus is mediated by P-selectin expressed on bound platelets, the anti-P-selectin antibody GA6 (1 mg per kg body mass) was infused into a baboon through the venous port in the shunt, 1 h after the infusion of ¹¹¹In-labelled-leukocytes and 1 min after the insertion of the Dacron graft into the shunt. The accumulation of ¹¹¹In-labelled-leukocytes within the shunt was depressed by the anti-P-selectin

antibody and the rate of leukocyte uptake was decreased by about 50%. No change in the platelet count in the blood was noted.

Similar results were obtained using GA6 Fab₂ fragments; the rate of leukocyte uptake was decreased by about 80%. As a control, the Fab₂ fragment (1 mg per kg body mass) derived from AC1.2, a non-inhibitory anti-P-selectin antibody¹, was shown to have no effect on leukocyte accumulation (Fig. 2b). These results indicate that anti-P-selectin antibodies inhibit the accumulation of leukocytes into platelet-rich thrombi *in vivo* and were confirmed in independent experiments on a different baboon. As in the previous animal, leukocyte accumulation within the Dacron graft was similar in the presence or absence of Fab₂ fragments of AC1.2 (Fig. 2c). In contrast, GA6 or its Fab₂ fragments (1 mg per kg body mass of each) caused a 75% reduction in leukocyte accumulation 120 min after introduction of flow within the shunt.

Although our anti-P-selectin antibodies do not interfere with any known *in vitro* platelet functions except heterotypic cellular adhesion^{1,3,17}, we confirmed that GA6 or its Fab₂ fragments did not alter the accumulation of platelets in the graft (Fig. 2d). Baboon platelets were labelled with indium-111 and infused into a baboon. The rate of platelet deposition and the number of platelets bound were similar in the presence and in the absence of GA6, and thrombocytopenia was not seen.

The Dacron graft was examined by scanning electron microscopy after each experiment. A graft obtained from an animal that was not infused with anti-P-selectin antibody contained leukocytes (Fig. 3a). In contrast, a graft obtained from an animal infused with GA6 (Fig. 3b) or GA6 Fab₂ (Fig. 3c) showed a reduction in associated leukocytes. An apparent decrease in erythrocytes associated with the grafts from animals infused with GA6 or its fragments has not been quantitated.

To investigate the physiological role of leukocytes bound within the thrombus, we examined the effect of GA6 on fibrin deposition *in vivo*. We used a radiolabelled fibrin-specific monoclonal antibody, T2G1s (ref. 18), which reacts with fibrin but not fibrinogen and is thrombus-specific, to detect and quantitate the kinetics of fibrin deposition *in vivo*¹⁹ (Fig. 4). When Fab₂ fragments of GA6 were infused, the amount of fibrin deposition

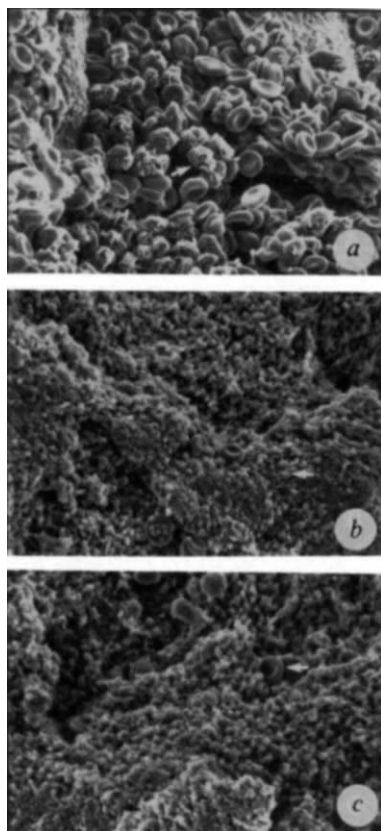


FIG. 3 Scanning electron micrographs of the surface of the lumen of the Dacron graft removed from the arteriovenous shunt. a, No anti-P-selectin antibodies. Arrow indicates a bound leukocyte. b, GA6. Arrow indicates a bound erythrocyte. c, Fab₂ fragments of GA6. Arrow indicates a bound erythrocyte. Micrographs were derived from Dacron grafts removed from baboon B after the completion of each experiment.

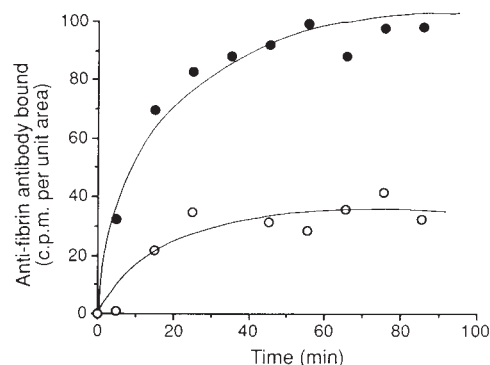


FIG. 4 Fibrin deposition on the Dacron graft and its inhibition by GA6 antibody. Data are from baboon A, with (○) or without (●) GA6 Fab₂ (2 mg per kg body mass).

METHODS. *In vivo* experiments were performed as in Fig. 2 except that cells were not labelled and radiolabelled anti-fibrin antibody-binding to the graft was monitored. The Fab' fragment of T2G1s was labelled with technetium-99m. The antibody (2 mCi) was injected through the venous connector of the shunt just before the initiation of flow, whereas, where indicated, GA6 Fab₂ was also injected simultaneously through the arterial connector during the first minute of flow. Images were obtained over the shunt using an Ohio Nuclear series 100 Gamma camera. A medium-energy, high-resolution, parallel-hole collimator was used with a 20% window. The kinetics of Tc-99m T2G1s antibody uptake by the graft and blood pool were derived from the radionuclide images. The activity was expressed as counts per min, normalized to injected dose and corrected for background and blood pool activity.

was reduced by 50–70%. In contrast, infusion of Fab₂ fragments of AC1.2 had no effect on fibrin deposition. These results indicate that leukocyte accumulation on the platelet-coated graft surface is associated with the deposition of fibrin to form the thrombus.

The main constituents of thrombi include fibrin, activated platelets and erythrocytes. Although leukocytes have been observed within thrombi, it was not clear whether circulating leukocytes are mechanically trapped by the polymerization of fibrin strands within the developing clot or through specific adhesive interactions between components of the thrombus, or both. On the basis of our results, we conclude that leukocyte accumulation in thrombi is an example of cellular adhesion. We suggest the following sequence of events. Platelets accumulate rapidly within the growing thrombus. Their adherence to the Dacron graft is probably mediated by von Willebrand factor. On platelet stimulation, the bound platelets degranulate and rapidly express P-selectin on the platelet surface. Circulating monocytes and neutrophils could then interact with the bound platelets through P-selectin, initiating a series of binding events that may lead to stable interaction of leukocytes with platelets within the thrombus. Our data are consistent with this model inasmuch as platelet deposition parallels leukocyte accumulation, and leukocyte-platelet interaction is blocked by anti-P-selectin antibodies. Furthermore, anti-P-selectin antibodies do not interfere with platelet-graft interaction.

The conversion of fibrinogen to fibrin by thrombin is the final event in the blood coagulation pathway. This pathway is initiated through the action of tissue factor which is expressed constitutively on non-vascular cells and can be induced on the surface of monocytes and endothelial cells²⁰. In the absence of tissue injury, with the concomitant exposure of blood to tissue factor on non-vascular cells, the mechanism for the activation of blood coagulation is uncertain. Such is the case in this baboon arteriovenous shunt model of experimental thrombosis. But the expression of tissue factor by graft-bound monocytes after monocyte activation represents a potential source of tissue factor. Indeed P-selectin increases tissue factor expression on monocytes (A. Celi, B.C.F. and B.F., unpublished results). It is possible that monocytes bound through P-selectin to adherent platelets on the graft, express tissue factor locally, and initiate and promote fibrin deposition on the graft. In our experiments, inhibition of leukocyte-binding to platelets on the graft by anti-P-selectin antibodies was associated with inhibition of fibrin deposition on the graft. We hypothesize that the presence of leukocytes in the growing thrombus contributes directly to fibrin deposition, but we presently have no data to demonstrate that fibrin generation is mediated by tissue factor on leukocytes in this experimental model.

Our results suggest that agents that inhibit P-selectin-leukocyte interaction *in vitro* will inhibit platelet-leukocyte interaction *in vivo*. Monocytes can be induced to express tissue factor and may play a role in thrombogenesis. Neutrophil-platelet interaction may be associated with transcellular metabolism of eicosinoids important to inflammation, thrombogenesis and wound healing. Furthermore, a mechanism for localization of leukocytes within the site of tissue injury may serve to contain the inflammatory and haemostatic process within these sites. A central role of P-selectin in inflammation and thrombosis is demonstrated. □

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Crystal structure of a Src-homology 3 (SH3) domain

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THE Src-homologous SH3 domain is a small domain present in a large number of proteins that are involved in signal transduction, such as the Src protein tyrosine kinase, or in membrane-cytoskeleton interactions, but the function of SH3 is still unknown (reviewed in refs 1–3). Here we report the three-dimensional structure at 1.8 Å resolution of the SH3 domain of the cytoskeletal protein spectrin expressed in *Escherichia coli*. The domain is a compact β -barrel made of five antiparallel β -strands. The amino acids that are conserved in the SH3 sequences are located close to each other on one side of the molecule. This surface is rich in aromatic and carboxylic amino acids, and is distal to the region of the molecule where the N and C termini reside and where SH3 inserts into the α -spectrin chain. We suggest that a protein ligand binds to this conserved surface of SH3.

Spectrin is a major component of the cytoskeleton that underlies the cell membrane⁴. The α - and β -subunits of spectrin are mostly made of 106-residue repeats. The SH3 domain is flanked by structural repeats in the middle of the 285K α -subunit^{5,6}. A plasmid expressing the SH3 domain in *E. coli* was constructed using the polymerase chain reaction with a complementary DNA clone⁵ encoding chicken brain α -spectrin (fodrin) as a template. The protein was purified and crystals grown as described in the legend to Fig. 1. The crystals are orthorhombic (space group $P2_12_12_1$, and unit cell dimensions $a = 34.2$ Å, $b = 42.4$ Å and $c = 50.2$ Å) and diffract X-rays beyond 2 Å. We obtained the best crystals at acidic pH, but the spectrin SH3 domain crystallizes in an isomorphous form at pH values between 3 and 9. The conformation of this protein is therefore not largely affected by pH.

The structure of the SH3 domain was solved by the technique of multiple isomorphous replacement including anomalous scattering information (MIRAS) (Table 1). A single successful heavy-atom reagent (K_2PtCl_6) was found. Data from two different soaking experiments using this compound showed different heavy-atom occupancy. It was possible to obtain an interpretable MIRAS map with these two datasets including their anomalous differences (Fig. 1b).

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The model has been refined to a crystallographic *R*-factor of 19.5% at 1.8 Å resolution, with good agreement to standard stereochemical parameters, and a 'free-*R*' value⁷ of 27.7%. The final model consists of 472 protein atoms, comprising residues 6–62 of the expressed sequence (Fig. 1a) and 38 solvent atoms. Electron density for residues 1–5 extends away from the molecule into solvent regions, but there is structural disorder. The rest of the domain forms a compact spherical structure (diameter ~30 Å) with a hydrophobic core and without any deep pocket. All the residues within this structure are well defined, except for the tripeptide Asn 47–Asp 48–Arg 49 and the C-terminal residue Asp 62, which are in weaker electron density and have high thermal parameters.

The secondary structure of the SH3 fold (Fig. 2) is formed from five antiparallel β -strands, with a long 19-residue interconnecting loop between the first two strands. The five strands form two orthogonal β -sheets, as in a β -sheet sandwich. Each sheet

consists of three strands, with one shared strand that begins in one sheet and continues in the other. If the five strands are termed a, b, c, d and e, then one sheet is formed from strands b–a–e, and the second sheet from strands b–c–d. The long loop connecting strands a and b forms a hairpin-like structure, but it lacks some of the characteristic hydrogen bonds of two adjacent antiparallel β -strands. The connection between strands d and e is formed by one turn of 3_{10} helix, which is initiated by the well conserved residue Pro 54. Between strand c and strand d there is a tight turn, such that residues 46 and 49 are hydrogen-bonded through main-chain atoms. We are unaware of a fold closely related to the SH3 fold, although a topological alignment is possible with some strands of the retinol-binding protein⁸.

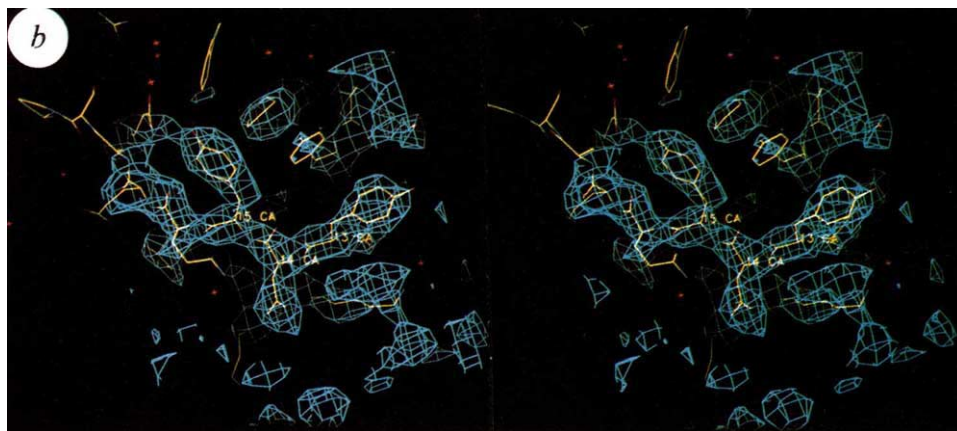
SH3 was first identified as a conserved sequence motif of about 60 residues in the cytoplasmic protein tyrosine kinases, phospholipase C- γ and the viral oncogene product Crk (refs 9–11). Apart from these proteins, SH3 has been found in a

FIG. 1 a, Sequence of the expressed

SH3 domain. Amino-acid sequence of SH3 domain in the chicken α -spectrin is shown in the upper line. The first methionine residue is not present in the authentic sequence⁵ but is added because of the expression of the domain using the pET3d vector²⁹. The consensus sequence for SH3 is shown in the bottom lines. The residues present in more than 52 (80%) of the 65 domain sequences^{3,19,20} are shown in single-letter amino-acid code. For instance, at position 22 this criterion holds for glutamate and aspartate together. The positions that have valine, leucine or isoleucine in 80% or more of the sequences are labelled with #. Position 52 (labelled with ϕ) has an aromatic side chain in 69% of the sequences. b, MIRAS electron density. Only one of the two alternative enantiomeric heavy atom positions gave rise to a phase set which, when used in map calculation, produced a clear solvent boundary. The refined model (near residues 13–15) is shown against the original 2.8 Å MIRAS electron density map, which is contoured at 1 σ . The connectivity of this density was further improved by solvent flattening using a protein envelope calculated from main-chain 'BONES' atoms traced automatically in the original MIRAS map. The program O (ref. 30) was used in combination with BONES atoms to provide an initial C α chain tracing. Main-chain and C β atoms were added to this automatically with the LEGO options of O, and the fit of the resulting polyalanine model was optimized. Phases calculated from this model were combined³¹ with the original MIRAS phases and used in calculation of a further map. In this map, all stretches of electron density were connected, and part of the model had to be retraced in the opposite direction. When this was done, model phases were again recombined, and side-chain electron density could be used to identify the sequence of the entire interpreted chain, starting from the Trp–Trp pair. Side chains were added using the O rotamer database, with some manual intervention. The resulting model extended from residue Leu 8 to residue Lys 60, with weak electron density for residues 47, 48, 49 and 50. This model was refined with the XPLOR³² package against a high-resolution dataset collected on beamline X31 at the Hamburg DESY facility (completeness to 1.8 Å = 96%, R_{merge} = 10.3%). To account for slight differences in cell dimensions, refinement was begun by rigid body procedure against this data set. Subsequently, refinement was pursued using alternating cycles of model rebuilding (with programs O and FRODO) and automatic refinement (using XPLOR and TNT³³). The model was extended to include the C-terminal residues Leu 61 and Asp 62, and two of the seven missing N-terminal residues (Lys 6 and Glu 7), to provide a total of 472 protein atoms; 38 water molecules were added to the model in electron density peaks at chemically feasible positions. The final three simulated annealing cycles, and many cycles of TNT refinement, were performed using a 90% subset of the available reflections to allow calculation of a free *R* value using the remaining 10%. The final conventional *R* factor

a

1	10	20	30	40	50	60
(M) DETGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWKVEVNDRQGFVPAAYVKKLD						
ALYDY	E#	GD	##	WW	G ϕ P	Y#
IFNF	D	E		Y		F



was 19.5% (5.0–1.8 Å, 5,627 reflections), with a free *R* value of 27.7% for the same resolution range; r.m.s. deviation from ideal bond lengths was 0.017 Å, and 2.7° from ideal bond angles. The r.m.s. deviation of side-chain CH1/CH2 angles from preferred values of a database³⁴ was 38°, and the r.m.s. difference in atomic B factors for covalently bonded pairs was 5.9 Å². Only Asn 47, which is in weak density, has ϕ/ψ values which are outside the accepted region for non-glycine residues in the Ramachandran plot.

METHODS. The 62-residue protein was soluble in *E. coli* BL21 (DE3). Bacterial cells were collected after 3 h of induction with isopropyl-thio- β -D-galactoside, suspended into 20 mM Tris–HCl, pH 8.2, buffer containing proteolytic inhibitors phenylmethyl sulphonyl fluoride (0.1 mM) and benzamidin (0.1%), and broken with a French press at 4 °C. SH3 was precipitated from the supernatant using ammonium sulphate between 50 and 75% saturation. Precipitated SH3 was only slightly soluble in the Tris buffer at pH 8.2. This buffer was therefore used to dissolve contaminating proteins, leaving SH3 in the pellet. SH3 was dissolved into 10 mM citric acid at pH 3.5 and purified using a Superdex-75 column, which was equilibrated with the same buffer and run with a Pharmacia FPLC instrument at room temperature. Crystallization was achieved using the hanging drop method at room temperature. The spectrin SH3 domain crystallizes in various conditions, the best crystals being obtained with vapour diffusion against a reservoir solution containing 90 mM sodium citrate/citric acid, 90 mM bis-Tris propane, 0.9 mM dithiothreitol, 0.9 mM EDTA and 0.9 mM Na₂S and 25–30% saturated ammonium sulphate (pH ~4); the protein (3 mg ml⁻¹ in 10 mM citric acid, pH 3.5) was initially mixed at 1:1 with this solution. The size of these crystals could be improved by macroseeding to 0.2 $\times$ 0.2 $\times$ 0.8 mm³. A heavy-metal derivative was obtained by soaking the crystals in this solution (adjusted to pH 4.7) containing 50% saturated ammonium sulphate and 10 mM K₂PtCl₆ for 2–3 weeks at room temperature.

FIG. 2 Topology of the spectrin SH3 domain. *a*, Schematic representation of the secondary structure identified using the DSSP package³⁵. Arrows represent β -strands labelled a, b, c, d and e, and the cylinder represents residues involved in a turn of 3_{10} helix. The numbers of the residues that form the secondary structure elements are shown within the arrows and cylinder. Numbers in parenthesis give the approximate position of some other residues. *b*, The $C\alpha$ trace is shown from a view similar to that indicated by the topology diagram. The figure was generated using the local program XRENDER (M.N.).

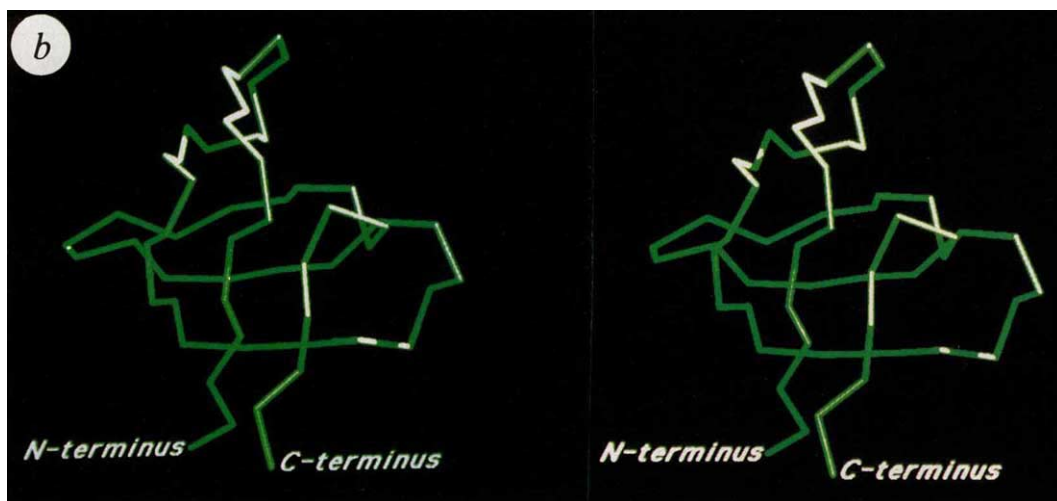


FIG. 3 Distribution of conserved residues in the SH3 domain. *a*, The main-chain trace (in yellow) of the crystallographically observed residues (6–62). Also shown are the side chains of residues 11–15, 22, 28–29, 41–42, 51–52, 54 and 57. These amino acids are the conserved residues identified in Fig. 1*a*. Phenylalanine 52, which represents aromatic residues conserved in this position in 69% of the sequences, is also shown. Note the four aromatic residues and the proline that form a continuous surface feature from left to right: Phe 52, Trp 41, Pro 54, Tyr 57 and Tyr 13. The N terminus (Lys 6) and the C terminus (Asp 62) are also labelled. *b*, CPK model. Conserved residues are shown in yellow, and the non-conserved residues are in blue. In the front-view figure, the presentation is the same as that used for *a*. In the back view, the model has been rotated 180° about the vertical axis. These diagrams were generated with the program XRENDER (M.N.).

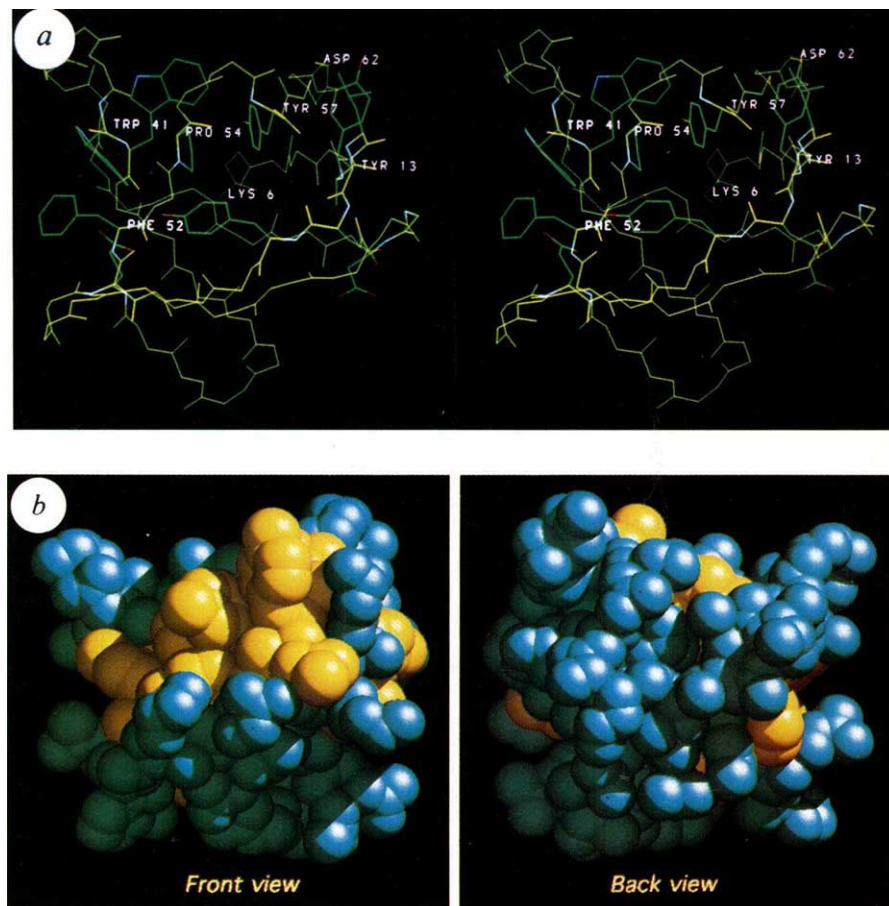


TABLE 1 Data used in solving the structure

Data statistics (space group $P2_12_12_1$)

Crystal	a (Å)	b (Å)	c (Å)	Maximum resolution (Å)	Completeness to maximum resolution (%)	$R_{\text{merge}}^{\dagger}$ (%)	Reflections to maximum resolution
FAST native dataset	34.1	42.4	50.0	2.6	89.3	6.6	2,205
1st platinum dataset	34.1	42.5	50.5	2.6	91.0	3.9	2,226
2nd platinum dataset	34.0	42.4	50.2	2.6	90.1	4.5	2,204

Heavy atom data (calculated with 2.8 Å data)	$R_{\text{diff}}^{\ddagger}$ (%)	x	y	z	Occupancy	B (Å ²)	R.m.s. phasing power§ (acentrics/centrics)
Dataset 1	24.1	-0.130	-0.076	-0.108	1.78	29.4	2.0/1.4
Dataset 2	15.9	-0.131	-0.076	-0.107	1.01	24.1	1.8/1.1

Data were collected on a FAST area detector mounted on an Elliot GX21 rotating anode generator. Data collection was controlled with the program MADNES, and profile fitting was done with the XDS package²⁶. The orthorhombic space group was identified using MADNES routines, and precession photography was used to determine that the space group was $P2_12_12_1$. Extensive trials produced a single heavy-atom derivative with an interpretable Patterson. The heavy-atom structure of this platinum derivative (see legend to Fig. 1b) could be solved by either SHELX, or the program VECSUM from the CCP4 package²⁷. Patterson calculations in increasing resolution shells showed that the isomorphous signal extended to better than 3 Å resolution, and the anomalous signal to 3 Å. A 3-Å SIRAS map, however, failed to produce a clear chain tracing, even after application of solvent-flattening using a reciprocal space implementation (CCP4) of the Wang procedure. Recollection of the derivative dataset under similar conditions were performed to improve the completeness of the reflections for which Friedel pairs were recorded. This new dataset (dataset 2) showed the same site, with somewhat different occupancy. Heavy-atom parameters for each of the two datasets were refined as for different derivatives using MLPHARE²⁸, which was also used to calculate phases. These two datasets gave an overall mean figure of merit of 0.73 for reflections to 2.8 Å and phasing power as specified in the table.

$$\dagger R_{\text{merge}} = \frac{\sum_h \sum_i |\bar{I}_h - I_{hi}|}{\sum_h \sum_i \bar{I}_h}$$

$$\ddagger R_{\text{diff}} \text{ (fractional isomorphous difference)} = \frac{\sum |FP - FPH|}{\sum FP}, \text{ where } FP \text{ and } FPH \text{ are the measured structure factors for the native and heavy-atom derivative}$$

crystals, respectively.

§ R.m.s. phasing power = r.m.s. (f_c)/r.m.s. (lack-of-closure error), where f_c is the calculated heavy-atom structure factor.

subunit of phosphoinositol-3-OH kinase, Ras-GTPase activating protein (GAP), myosins IB and IC, several yeast proteins involved in cell polarity and morphogenesis, the nematode cell-signalling protein Sem5 and in many others³. It is often accompanied by another small protein domain called SH2, a 100-residue domain that recognizes and binds peptides containing a phosphorylated tyrosine and which is essential to interactions between many receptors and signalling proteins^{1,2,12,13}. The three-dimensional structures of SH2 and SH3 are not related. SH2 is made of a central antiparallel β -sheet which is flanked by two amphipathic helices¹⁴⁻¹⁶. The recognition site for the phosphotyrosine peptide is a flat surface perpendicular to the β -sheet. This surface contains a binding pocket for phosphotyrosine¹⁴.

Unlike SH2, the function of SH3 is still unclear. Circumstantial evidence—its presence in proteins that are permanently or transiently bound to the cell membrane—suggests that SH3 might interact with components of the membrane or cortical cytoskeleton^{2,3}. A protein called 3BP-1 has been identified from a mouse B-cell library that binds to the SH3 domain of Abl tyrosine kinase¹⁷. The deduced partial sequence of 3BP-1 is homologous to the human Bcr protein (the product of the break-point cluster region gene of the Philadelphia chromosome translocation) and to neuronal protein n-chimaerin. All three proteins have an amino-acid stretch that is very similar to a short peptide sequence of a human Rho-GAP¹⁸. This homology and the demonstrated GAP activity of Bcr and n-chimaerin¹⁸ have led to the proposal that the SH3 domain of Abl might bind to a GTPase-activating protein, which implies a functional association between SH3 domains, GAPs and G proteins such as Rho or Rac¹⁷.

SH3 has no particular preference for its location in the host protein sequence. It seems to function as an independent unit. In some cases it is present at the extreme of the N or C terminus, which has helped to define the domain boundaries³. SH3 is found both in enzymes and in proteins that may have no catalytic activity. It is usually present as a single copy but Sem5 and its human homologue GRB2, for instance, have two copies of SH3. Sem5 and GRB2 are examples of proteins solely constructed of SH2 and SH3 domains^{19,20}.

The most striking feature of the SH3 structure is that the conserved residues of SH3 sequences (Fig. 1a) are close to each other in the structure and form a smooth patch on the protein surface (Fig. 3). The only exception is Trp 42, which is in a hydrophobic pocket in the interior of the molecule. The conserved surface is rich in aromatic residues and contains Tyr 13, Tyr 15, Trp 41, Phe 52 and Tyr 57. It has three carboxylic acids, Asp 14, Glu 22 and Asp 29. Glutamic acid 22 forms a hydrogen bond to the phenolic hydroxyl group of Tyr 15. Glycines 28 and 51 as well as Pro 54 are also located on this surface. Mutations Gly 51 → Arg and Pro 54 → Leu inactivate the function of Sem5 (ref. 19). Two point mutations in the Src-tyrosine kinase that affect residues 18 and 19 in the a-b loop cause cell transformation²¹. Two alternatively spliced forms of neuronal Src are known, in which insertions of six and seventeen amino acids in SH3 seem to cause increased tyrosine kinase activity and altered transforming potential²²⁻²⁴. These insertions take place after position 38 in the loop between strands b and c.

The smooth conserved surface of the molecule is likely to be a binding site for a large molecule, probably another protein. It is rich in aromatic residues, which are often involved in protein-protein interactions²⁵. The amino-acid residues in this putative binding site are not always completely conserved (Fig. 1a). This may indicate that SH3 domains recognize a family of related proteins in different species or tissues. In the proteins that contain multiple SH3 domains, these are not identical but, for instance, have variations on the N-terminal Ala-Leu-Tyr-Asp-Tyr (ALYDY) motif^{3,19,20} (residues 11-15 in Fig. 1a). A possible explanation for this internal variation is that multi-SH3-domain proteins are able to recognize several related proteins. □

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Structure of hisactophilin is similar to interleukin-1 β and fibroblast growth factor

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THE fast reaction of the actin-based cytoskeleton in motile cells after stimulation with a chemoattractant requires a signal-transduction chain that creates a very specific environment at distinct regions beneath the plasma membrane¹. *Dictyostelium* hisactophilin, a unique actin-binding protein, is a submembranous pH sensor that signals slight changes of the H⁺ concentration to actin by inducing actin polymerization and binding to microfilaments only at pH values below seven². It has a relative molecular mass of 13.5K and its most unusual feature is the presence of 31 histidine residues among its total of 118 amino acids. The transduction of an external signal from the plasma membrane to the cytoskeleton is poorly understood. Here we report the protein's structure in solution determined by nuclear magnetic resonance spectroscopy.

The nuclear Overhauser effect intensities of the three-dimensional nuclear Overhauser spectra^{3,4} were used directly in the calculations⁵. The overall folding of hisactophilin is similar to that of interleukin-1 β ^{6–9} and fibroblast growth factor¹⁰, but the primary amino-acid sequence of hisactophilin is unrelated to these two proteins.

Protonated and ¹⁵N-labelled hisactophilin was expressed in *Escherichia coli* using the pIMS expression vector and purified essentially as described². Purification and biochemical properties of the bacterially expressed protein were indistinguishable from *Dictyostelium* hisactophilin, except that the *E. coli* protein contains an insertion of four amino acids, Gly-Glu-Phe-Gly, after the initial Met. NMR data showed that hisactophilin has a stable conformation at pH values from 4.7–8.0; below pH 4.7 the NMR spectra are typical of a random-coil structure. Hisactophilin can be refolded, however, to its native state by increasing pH. We chose to study hisactophilin at pH 5.7 and 6.7, the points at which hisactophilin binds to actin. The data at pH 6.7 are considered here but the conformation at pH 5.7 is very similar. The NMR spectra of hisactophilin were assigned with the homo- and heteronuclear two-dimensional and three-dimensional methods^{11–13}. The topology of the secondary structure elements is summarized in Fig. 1; it can be seen that hisactophilin consists of 12 β -strands connected by turns and loops.

The three-dimensional structure of hisactophilin was calculated from 2,541 intensity constraints (without transforming

FIG. 1 Schematic representation of the polypeptide arrangement of hisactophilin. The amino acids are numbered according to the sequence of hisactophilin from *D. discoideum* because there were only trivial NOEs connecting to the insertion segment of the *E. coli* protein (see text). A heavy line connects successive amino acids (circles) in the polypeptide chain. Inter-main-chain hydrogen bonds, shown by thin lines, were selected according to ref. 11. Histidines are represented by shaded circles and glycines by hatched circles. Individual strands of the β -barrel are illustrated as the spokes of the wheel, with turns and chain termini at the hub. Longer loops that cover the other side of the barrel are drawn along the outer rim. This design was chosen to emphasize the internal 3-fold symmetry of the molecule.

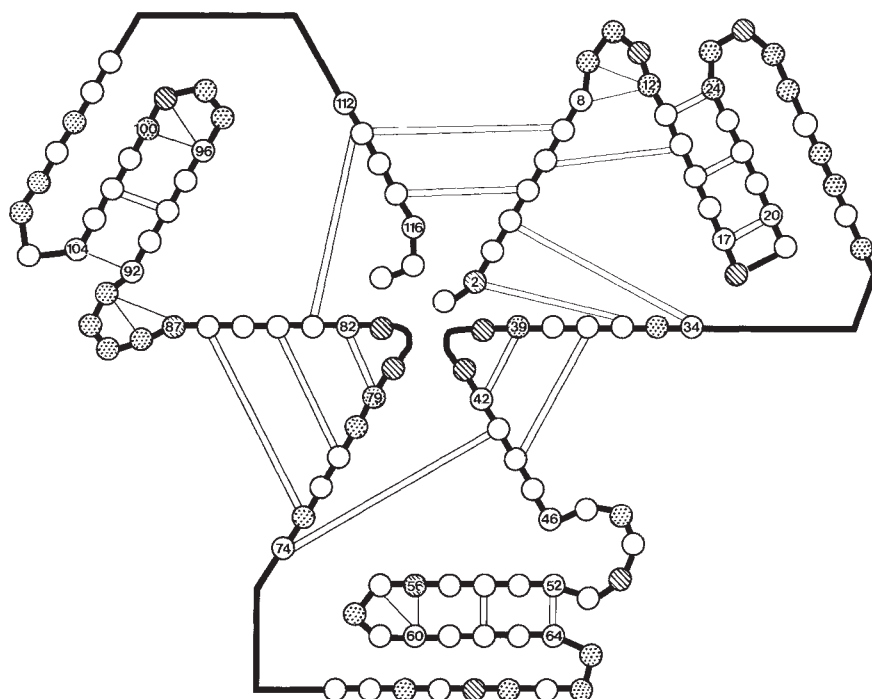


TABLE 1 Secondary structure of hisactophilin and sequence alignments of the residues in hisactophilin and IL-1 β that are structurally equivalent

	Barrel		Hairpins		Barrel
S1	Strand β_1	Hairpin 1 (β_2)	Hairpin 1 (β_3)	Strand β_4	
IL-1 β	¹¹ R D	¹⁶ K S L* V M	²⁹ E L* K A L	⁴² F S M	
His	² G N R A F K S	¹² H F L* S A E	²⁰ A V* K T H	³⁴ F H V E N H	
S2	Strand β_5	Hairpin 2 (β_6)	Hairpin 2 (β_7)	Strand β_8	
IL-1 β	⁵⁷ P V A L G	⁶⁸ Y L* S C V	⁷⁷ —	¹⁰¹ F N K I E I	
His	⁴² K V A L K	⁵² Y L* S I G	⁶⁰ Q V Y L* S	⁷⁴ F H L E H H	
S3	Strand β_9	Hairpin 3 (β_{10})	Hairpin 3 (β_{11})	Strand β_{12}	
IL-1 β	¹⁰⁹ K L E F E S	¹²¹ Y I* S T S	¹³¹ —	¹⁴⁵ D F	
His	⁸² K V S I K G	⁹² Y I* S A D	¹⁰⁰ H V* S T K	¹¹² T F E E I	
His	Turn β_{1-2} (I', I')	Turn β_{2-3} (II', II')	Loop $\beta_{3,4}$	Turn $\beta_{4,5}$ (-, II')	
S1	⁹ H H G H	¹⁷ E G E A	²⁹ H G H H D H H T H	³⁹ H G G K	
His	Turn β_{5-6}	Turn β_{6-7} (-, III')	Loop $\beta_{7,8}$	Turn $\beta_{8,9}$ (-, II')	
S2	⁴⁸ H C G K	⁵⁷ D H K Q	⁶⁵ H H L H G D H S L	⁷⁹ H G G K	
His	Turn β_{2-10} (III', III')	Turn β_{10-11} (III', III')	Loop $\beta_{11,12}$	—	
S3	⁸⁸ H H H H	⁹⁶ D H H G	¹⁰⁵ E H H D H D T		

The alignment was done by least-squares superposition of the C α s of the fragments of the two proteins with the program WHAT IF. For IL-1 β , residues for which mean deviations of the C α s from the C α s of hisactophilin were smaller than 6.32 Å are listed. The type of a turn in hisactophilin is indicated in parentheses in cases when the NMR determination (by NOE patterns) was unambiguous; a second entry categorizes the turns according to the definition of Kabsch and Sanders²⁰. Asterisks indicate residues between the hairpins that are critical for the packing of the hairpin triplet (see text).

them into distance constraints⁴). They were derived from the three-dimensional nuclear Overhauser effect (NOE) intensities of the three-dimensional nuclear Overhauser spectra (3D-NOE-NOE jump-return) experiment¹⁴ with both mixing times of 100 ms. From this number, 1,150 connectivities were derived from the 3D-NOEs on the N₁ and N₂ plane^{4,15}. The intensity constraints were supplemented with 27 ϕ backbone angle con-

straints derived from the two-dimensional *J*-correlated (2D COSY) spectrum¹¹. Eighty non-NOE distances and 45 hydrogen-bonding constraints were additionally used in the calculations¹⁶. The calculational protocol was similar to that used in the model calculations of a trypsin inhibitor, CMTI-I, and are described in detail in ref. 5. A floating chirality method in the simulated annealing stage of the calculations was used to obtain

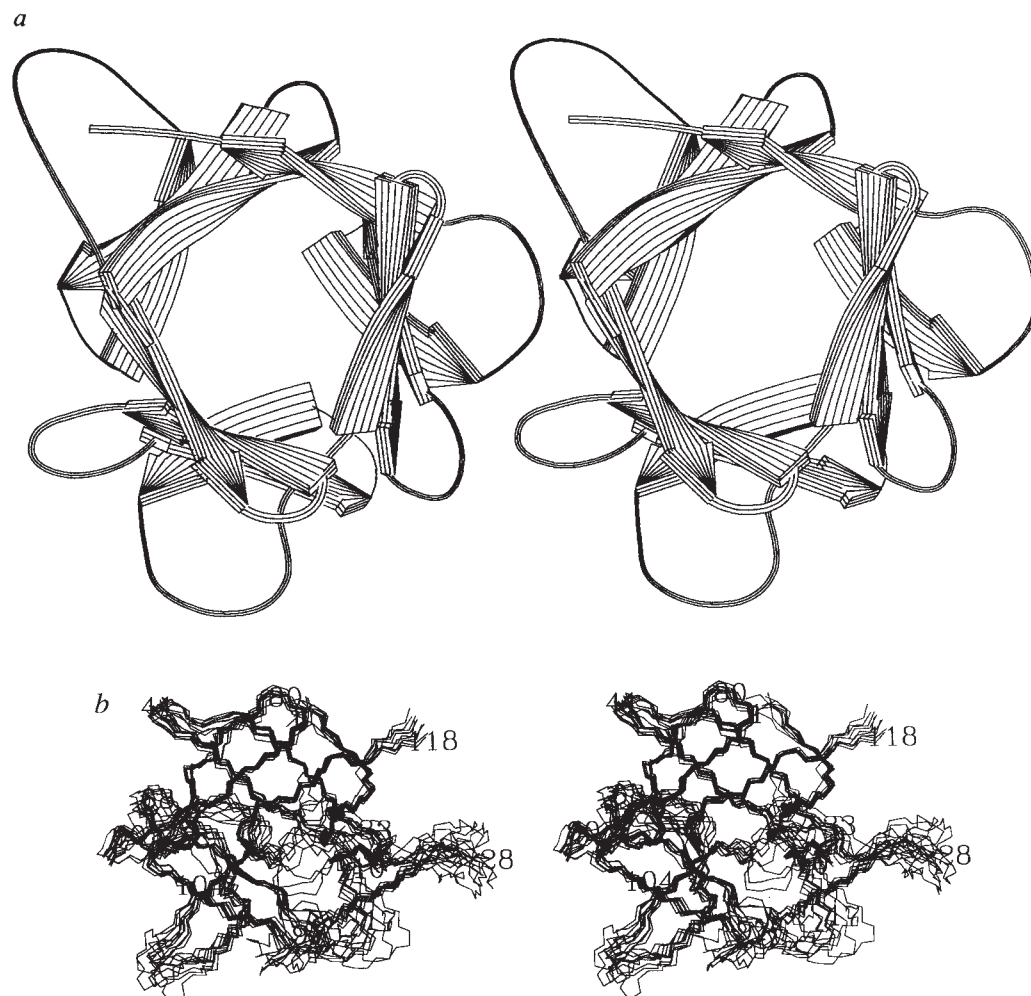


FIG. 2 a, Ribbon drawing of hisactophilin. b, Stereoview of the backbone atoms (N, C α , C) of 10 hisactophilin structures best fitted to all amino acids residues except residues 1, 25-32, 47-50, 55-59, 66-69, 96-100 and 108. For clarity, 10 structures were randomly chosen out of 20.

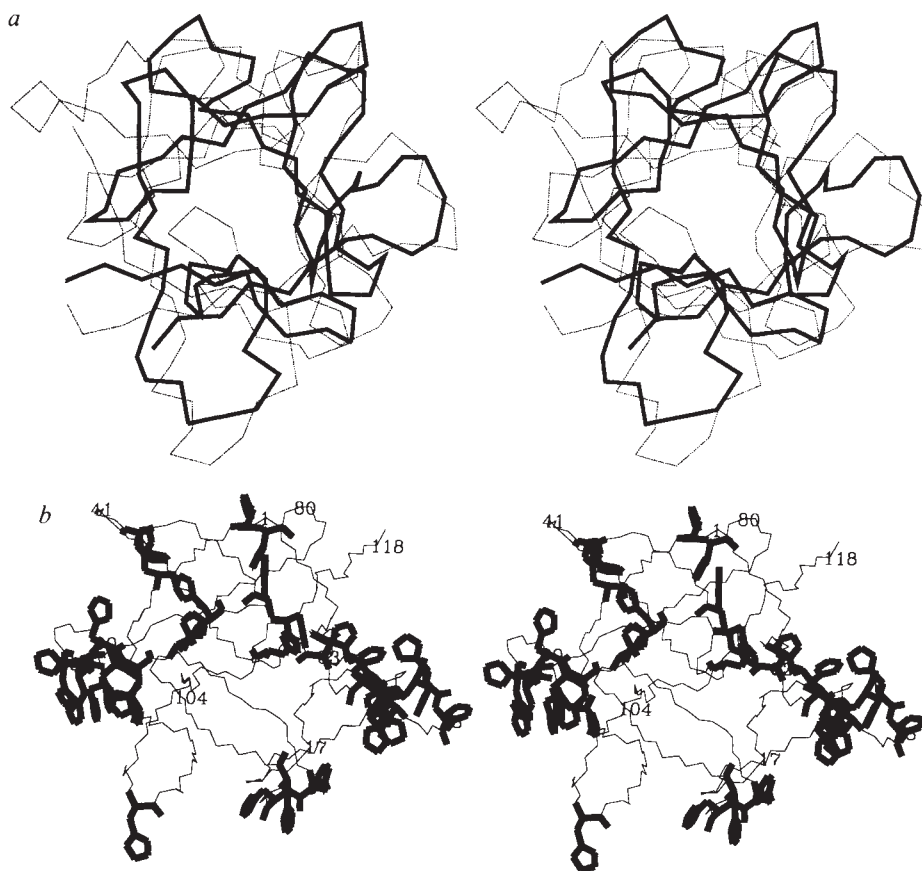


FIG. 3 *a*, Superposition of the C α traces of hisactophilin (solid line) and IL-1 β (broken line). *b*, Stereo picture of C α tracing of hisactophilin with side chains of histidine residues.

stereospecific assignments at prochiral centres^{4,16}. A total of 20 structures were calculated by a hybrid distance geometry-dynamical simulated annealing method¹⁶. All 20 structures satisfied the experimental constraints (the translation of the intensity violations into the distance violations is described in ref. 5). The structures also showed small deviations from idealized covalent geometry (bonds, angles and impropers), and had negative Lennard-Jones van der Waals energies. The averages of the r.m.s. differences for heavy atoms in β -sheets among the 3D-NOE-NOE structures were 0.84 ± 0.14 Å for the backbone atoms and 1.75 ± 0.18 Å for all atoms. The corresponding r.m.s. difference averages for heavy atoms in the loops were 2.3 ± 0.9 Å for the backbone atoms and 3.5 ± 1.0 Å for all atoms.

A ribbon drawing and a superposition of 10 structures of hisactophilin is shown in Fig. 2. The backbone of hisactophilin within the β -sheets is very well determined. The turns and loops show greater variability, with the loop between residues 26–33 having the highest variability. For many residues in this segment there were no NOEs at all in the NOE spectra, whereas strong *J*-peaks could be seen in the TOCSY (total correlation spectroscopy)¹¹ spectra. It is therefore postulated that this variability is associated with high flexibility of the loops. All glycines and histidines, except for His 35, His 75 and His 78, are located in the loops or turns (Fig. 1 and Table 1) whose atomic r.m.s. differences for the backbone atoms are larger than 1.6 Å.

A characteristic feature of the structure is the presence of 12 antiparallel β -strands arranged around a 3-fold axis of symmetry (Fig. 1 and Table 1). The molecule consists of three similar units (S1, S2 and S3; Table 1), each containing two pairs of antiparallel β -strands (Figs 1 and 2). Each unit has a β - β -loop- β motif. The first and fourth β -strand of one unit make up one β -sheet. These three β -sheets, one for each unit, form a barrel structure (strands β_1 , β_4 , β_5 , β_8 , β_9 and β_{12}). The other three pairs of β -strands (strands β_2 , β_3 , β_6 , β_7 , β_{10} and β_{11}) cover one end of the barrel (hairpins 1–3 in Table 1). The side chains of most of the hydrophobic residues are located inside the barrel.

A search for structure similarities to the known protein folds with the program WHAT IF¹⁷ revealed that the three-dimensional structure of hisactophilin is similar to that of interleukin-1 β (IL-1 β)^{6,9} and fibroblast growth factors (FGFs)¹⁰ (Fig. 3*a*). A detailed analysis of the 'IL-1 fold' (β -trefoil fold) has been done¹⁸. A comparable degree of structural similarity without sequence homology was previously observed with actin, hexokinase and the N-terminal 45K fragment of the heat-shock cognate protein, HSC70 (ref. 19). But although the primary amino-acid sequences of hisactophilin, IL-1 β , and FGFs are unrelated, the residues responsible for stabilization of the barrel are conserved (Table 1). The six-stranded barrel is stabilized by the tight inside-packing of large hydrophobic residues in the interior which are large enough to be close to the barrel axis. For example in interleukin-1 β , the critical interior residues are Phe 42, Phe 101 and Phe 146, which from the sequence alignment in Table 1 correspond to Phe 34, Phe 74 and Phe 113 of hisactophilin. Thus the conservation of these residues is remarkable. The barrel is covered on one side by three hairpins packed together in a triangular arrangement called the hairpin triplet for IL-1 β ¹⁸. The residues responsible for the packing of this triplet are also highly conserved in hisactophilin (Table 1). Without exception, these residues are Leu, Val or Ile. The only major difference between the IL-1 β and hisactophilin is that the network of interhairpin hydrogen-bonds is less extensive in hisactophilin than it is in IL-1 β . Also, the top surface layer of residues in the hairpin triplet is more 'open' in hisactophilin than it is in IL-1 β (Fig. 3*a*). At present, there are not enough data to speculate about any possible evolutionary relation between the two proteins. But because the two proteins play different biological roles it may be that the β -trefoil fold represents an energetically favourable folding unit that is not from a common ancestor.

The structure of hisactophilin suggests strongly how this protein functions in the living cell. Ninety per cent of all histidine residues (28 histidines) are located in loops and turns at the

surface of the molecule. These histidines were estimated from NMR titrations to have very similar pK_a values. A slight shift of the intracellular pH creates either a large number of positive charges or leaves the protein essentially uncharged. Considering the impact of polycations like polylysine on actin polymerization *in vitro*, it is evident that hisactophilin binds to actin in its polycationic form. Furthermore, the uneven distribution of histidines in two opposite patches in the hisactophilin molecule (Fig. 3b) explains the protein's ability to bind along actin filaments and to form large filament bundles. □

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Stable dye–DNA intercalation complexes as reagents for high-sensitivity fluorescence detection

Alexander N. Glazer and Hays S. Rye

Fluorescent intercalation complexes of certain polycationic ligands with double-stranded DNA provide a new class of multichromophore labels for fluorescence assays.

THE term 'intercalation' was introduced by Lerman in 1961¹ to describe the insertion of planar aromatic (or heteroaromatic) compounds between adjacent base pairs (bp) of double-stranded DNA (dsDNA). Intercalative binding follows the 'neighbour-exclusion principle' where every second site along the double helix remains unoccupied². In 1990, we reported the finding that complexes of ethidium homodimer (EthD) with dsDNA, preformed at a ratio of one dimer per 4 to 5 base pairs, were stable to electrophoresis on agarose gels. This allowed fluorescence detection and quantitation of DNA fragments with picogram sensitivity after separation in complete absence of background stain³. Here, we describe observations that show that complexes of dsDNA with various other fluorescent polycationic intercalators are also very stable and can replace radioisotopes in high-sensitivity DNA detection and multiplex restriction fragment mapping. Moreover, such dye–dsDNA complexes offer unique opportunities as generally applicable alternative multichromophore reagents in widely used fluorescence assays.

Polyfunctional intercalators

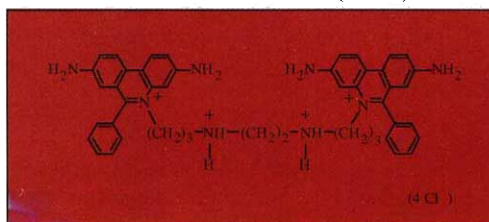
The binding constants of monointercalators to dsDNA are 10^5 – 10^6 M⁻¹ in low salt buffers. Compounds with two potential interca-

lating moieties in a single molecule have been investigated with the expectation that these would have association constants with dsDNA of 10^{10} – 10^{12} M (refs 4,5). Such expectations were at least partially fulfilled for several bisintercalators. The binding affinity was found to be further enhanced by utilizing suitably chosen cationic interchromophore linkers⁶. The crystal structure of the complex of the antitumor agent ditercalinium with double-stranded

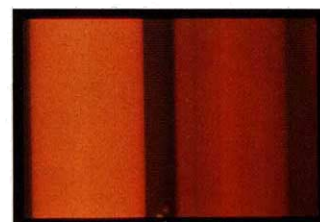
tetramer [d(CGCG)]₂ (Fig. 1)⁷ exemplifies the binding of such compounds to dsDNA.

In 1978, Gauguier *et al.*⁸ described a polyamine-bridged dimer (Fig. 2) of the familiar monointercalator ethidium. In 0.2 M Na⁺, EthD bound to dsDNA almost 10³ times more strongly than ethidium with a stoichiometry of one homodimer per four base pairs. On binding to dsDNA, the fluorescence quantum yield (*Q_F*) of the dimer increased 40 fold independent of base com-

Ethidium homodimer (EthD)

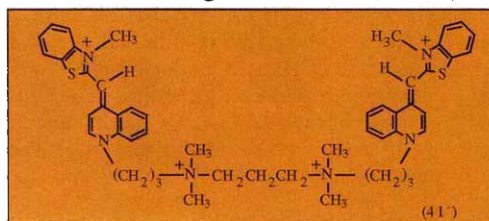


$$F_{\text{bound}}/F_{\text{free}} = 40$$

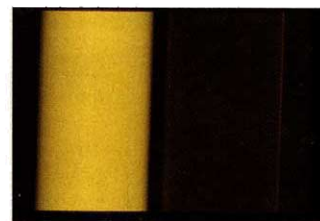


EthD-DNA EthD

Thiazole orange homodimer (TOTO)

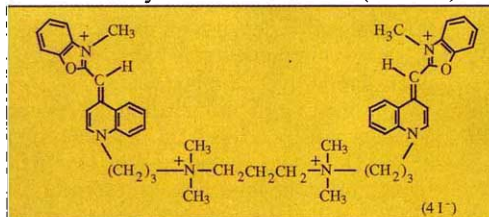


$$F_{\text{bound}}/F_{\text{free}} = 1,100$$



TOTO-DNA TOTO

Oxazole yellow homodimer (YOYO)



$$F_{\text{bound}}/F_{\text{free}} = 3,200$$



YOYO-DNA YOYO

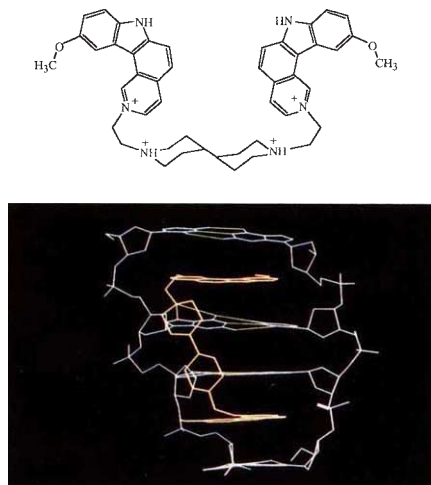


FIG. 1 The structure of ditercalinium and the crystal structure of the [d(CGCG)]₂-ditercalinium complex⁷.

FIG. 2 Structures of ethidium homodimer (EthD), thiazole orange homodimer (TOTO) and oxazole yellow homodimer (YOYO). The background for each structure approximates the colour of the free dye in aqueous solution. The fluorescence enhancement of each dye upon binding to dsDNA is given below each structure. The panels to the right of each structure show a fluorescence image of a pair of capillary tubes filled with the dye in the absence (right tube) and the presence of calf thymus DNA (100 µg ml⁻¹; left tube) at a molar ratio of base pairs to dye of 5:1.

position or nucleotide sequence⁹. Le Pecq and coworkers¹⁰ exploited the tight binding of EthD to develop a solution fluorescence assay for dsDNA at concentrations down to ng per ml. This work led us to examine the stability of EthD-dsDNA complexes to electrophoresis with the encouraging results noted above.

The observations on the EthD-dsDNA complexes³, together with earlier reports of stability to electrophoresis of complexes of dsDNA with the antibiotic luzopeptin (a cyclic depsipeptide that contains two 3-hydroxyquinolonic acid chromophores per molecule)¹¹, and with di-9-acridinylamine¹², suggested that high affinity binding of bisintercalators to dsDNA is a property shared by ligands differing widely in structure and prompted a search for fluorescent bisintercalators with more favorable spectroscopic properties than EthD. Such compounds would both increase the sensitivity of dsDNA detection and allow simultaneous detection of complexes of dsDNA with different dyes.

Asymmetric cyanine dyes

Lee *et al.*¹³ reported that the asymmetric cyanine dye, thiazole orange (TO), with a 14-fold higher absorption coefficient at ~500 nm than ethidium, bound to dsDNA with a stoichiometry characteristic of mono-intercalators, one TO per two base pairs, with a strong enhancement of fluorescence on binding. These findings were confirmed and developed further by Rye *et al.*^{14,15}, to show that the fluorescence enhancement of TO on binding to dsDNA is an extraordinary ~19,000 fold. The very low fluorescence of free TO allows detection of picogram quantities of dsDNA on gels¹⁴ or during capillary zone electrophoresis¹⁶ in the presence of 10^{-6} – 10^{-7} M free dye.

Dimers of TO and of oxazole yellow (an analogue of thiazole orange with an oxygen in place of the sulphur) connected by a bis-cationic linker¹⁷, designated TOTO and YOYO, respectively (for structures, see Fig. 2), bound to dsDNA with an affinity similar to that of EthD and, like TO, showed large fluorescence enhancements on binding (Fig. 2)¹⁵. For these dyes, as for EthD, the Q_F for the bound dyes was independent

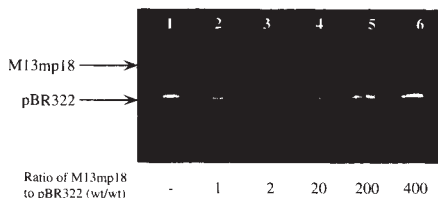
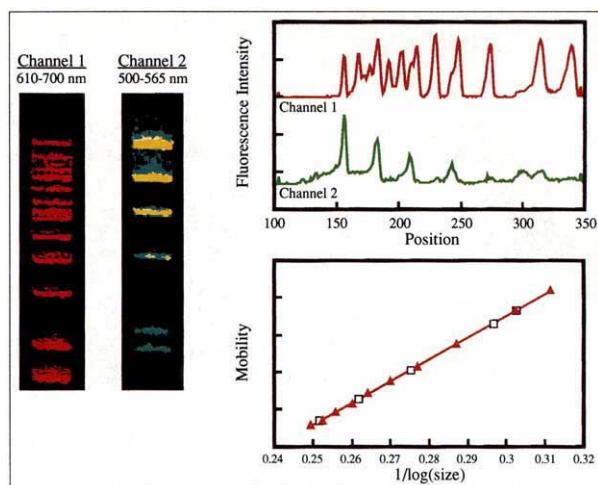


FIG. 3 Migration of TOTO between dsDNA molecules. Fluorescence image of a gel loaded with 250 pg of linearized pBR322 complexed with TOTO at a molar ratio of base pairs to dye of 5:1. For lanes 2–6, increasing amounts (as indicated below the image) of uncomplexed, linearized double stranded M13mp18 DNA were incubated for 30 min with the prelabelled pBR322 sample before loading on the gel.

FIG. 4 Multiplex dsDNA fragment detection and size determination. The fluorescence image from each of two detection channels of a single lane of an agarose gel loaded with a mixture of 1-kb ladder DNA prelabelled with EthD (8 ng at 10 bp per dye) and λ /HindIII DNA prelabelled with TOTO (1 ng at 20 bp per dye) is shown at left. The emission of the EthD complex was detected in channel 1 and that of the TOTO complex in channel 2. The upper plot to the right shows the fluorescence intensity of each channel as a function of the linear position down the gel. The lower plot shows a size-calibration curve calculated from the EthD-stained 1-kb ladder bands (red triangles) and the positions of the lower four bands of the TOTO-stained λ /HindIII pattern (open squares). Calculated sizes for the λ /HindIII fragments are within 2–7% of the actual values.



of the fractional saturation of the binding sites on the dsDNA.

Pre-staining and detection

Recent development of a laser-excited, confocal fluorescence gel scanner^{3,18} allowed full exploitation of the advantages of the 'pre-staining' approach. This two-colour scanner utilizes an epi-illumination format where the laser is focused on the sample by a microscope objective and the fluorescence is gathered by the same objective followed by confocal detection. This scanner is similar to that described recently for the detection of end-labelled DNA in capillary array electrophoresis^{19,20}. In detecting DNA sequencing gels, the scanner was shown to have a limiting sensitivity of ~10 attomoles of DNA 5'-labelled with a fluorescein-type fluorophore and a spatial resolution of $10 \mu\text{m}^2$. Pre-staining allowed post-electrophoresis detection with this scanner of dye-dsDNA complexes on gels with sensitivity similar to that attainable with radioisotopes^{3,15}.

Properties and applications

The most direct application of such complexes is to the sensitive fluorescence detection, sizing, and quantitation of dsDNA fragments separated on gels. The success of this application depends on the recognition of several properties peculiar to dsDNA-intercalation dye complexes.

The binding of an intercalator between adjacent base pairs results in local distortion of the DNA and an increase in base-pair separation at the intercalation site from 3.4 to 6.8 Å with extension and stabilization of the double helix. Moreover, with cationic ligands, the overall negative charge on the DNA is reduced in proportion to the number of ligand molecules bound. As anticipated, these effects lead to shifts in electrophoretic mobility. Surprisingly, with EthD, TOTO, and YOYO, the mobility shift is most pro-

nounced when comparing dsDNA fragments without ligands with those carrying relatively few dye molecules. For complexes containing more than one dimer per 50 base pairs, the variation in mobility with site occupancy is small. For example, taking the mobility of a 6.5-kb fragment at an EthD-to-base pair ratio of 1:40 as 1.0, the mobilities of this fragment at higher EthD-to-base pair ratios of 1:20 and 1:4 are 0.99 and 0.90, respectively³.

Because of the dependence of mobility on site occupancy, successful sizing of DNA fragments requires knowledge of the DNA concentration so that an appropriate amount of dye can be added. Knowledge of the DNA concentration is also important if intermolecular crosslinking of DNA fragments by the bisintercalators is to be avoided. Such artifact bands are seen at dsDNA concentrations in excess of $2 \mu\text{g ml}^{-1}$ at near site-saturating levels of bisintercalators. We have developed a solution assay, utilizing TOTO and YOYO, which gives a linear fluorescence response over a dsDNA concentration range of 0.5–100 ng ml⁻¹ and which requires as little as 25 pg of dsDNA²¹.

Another consideration is that some migration of dye from a dsDNA-dye complex to unlabelled dsDNA does take place (Fig. 3). However, when the occupancy decreases to ~80% for TOTO and YOYO, or to ~50% for ethidium dimer (assuming a ratio of 4 base pairs:dimer at saturation), there is no further migration of dye independent of the excess of unlabelled over labelled dsDNA.

Under experimental conditions that take the above constraints into account, plots of the relative electrophoretic mobility of a variety of ds-DNA fragments prelabelled with EthD, TOTO, or YOYO against fragment size are linear, as are the plots of fluorescence intensity against DNA concentration in base pairs^{3,15,22}, leading to the conclusion that the binding of these dyes does not show strong sequence specificity.

Multiplexing experiments were performed in which two ladders of DNA restriction fragments were each separately labelled with a different bisintercalator, mixed and run together in the same lane of an agarose gel. At the end of electrophoresis, scanning the gel with simultaneous two-colour fluorescence detection allowed sizing of the fragments in one of the ladders relative to those in the other ladder. Such an experiment is illustrated in Fig. 4. The multiplexing procedure allowed accurate sizing on agarose gels of dsDNA fragments ranging from 600 to 23,000 bp.

In other applications, labelling with the bisintercalators allowed detection of dsDNA products of the polymerase chain reaction with a sensitivity comparable to that obtainable with radioactivity. We have also found that interaction of binding proteins with dsDNA-dye complexes can be studied with high sensitivity.

Prospects

As outlined above, stable dsDNA-fluorophore complexes can be formed to contain anywhere from several to several thousand fluorophores each, as desired. Under suitably controlled conditions, these complexes do not transfer dye to other nucleic acids or to proteins. An important property of these complexes is that their fluorescence emission intensity is a linear function of the number of intercalated dye molecules. As high sensitivity fluorescence detection apparatus^{18,20} becomes more generally available, the ability to use dyes to replace radioactivity for sensitive detection

of DNA will become increasingly valuable.

Dye-dsDNA complexes represent a novel family of fluorescent labels with a wide range of spectroscopic properties, whose composition, structure and size can be tailored to particular applications. DNA molecules can be readily derivatized to attach biotin, digoxigenin, or any of a number of other substituents that can be recognized by avidin or antibodies. Such derivatized DNA molecules loaded with dye may allow detection at much higher sensitivity in numerous applications (for example, immunoassay, fluorescence *in situ* hybridization of chromosomes) that currently use other fluorescent labels.

Probes with a double-stranded region (to provide intercalation sites) and a single-stranded region to allow recognition by hybridization of specific target sequences offer another approach to the generation of versatile fluorescent labels. Development of conditions that allow clear discrimination between the binding of bisintercalators to single- and double-stranded nucleic acids is an essential prerequisite to the use of such probes and is under study. □

Alexander N. Glazer and Hays S. Rye are in the Division of Biochemistry and Molecular Biology, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA. This research was supported by the Office of Energy Research, Office of Health and Environmental Research of the US Department of Energy. Hays S. Rye is a recipient of a predoctoral fellowship from

the Department of Health and Human Services Training Grant. We thank Richard A. Mathies for valuable discussions and Loren D. Williams for the stick drawing of the [d(CGCG)]₂-ditercalinium complex. For more information, fill in reader service number 100.

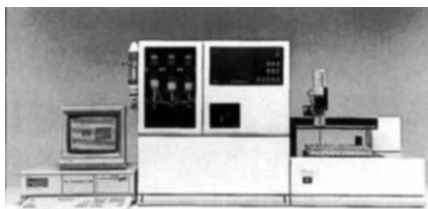
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The gene machine

Featured this week — DNA sequencing and analysis software, human genomic mapping probes and an enzyme-linked assay for the identification of amplified target DNA.

NOVAGEN announces the immediate availability of **rat embryos ready for *in situ* hybridization** (Reader Service No. 101). The line of products qualified for *in situ* hybridization also includes mouse embryos and adult mouse and rat tissues. The fixed tissues are embedded in paraffin and sectioned at a thickness of 7 µm.

Millipore has introduced a new line of **PEG-PS peptide synthesis supports** for use with the company's 9050 Plus peptide synthesizer (Reader Service No. 102). The company says that the supports are compatible with a wider range of solvents than other supports and routine cycle times of 16 min are possible when they are used on the 9050 Plus. PEG-PS supports uniformly incorporate a derivatized polyethylene glycol (PEG) spacer between functional groups on the polystyrene (PS) backbone and attachment point of the peptide being synthesized.



PEG-PS peptide synthesis supports.

sized. This structure, Millipore says, creates a rugged particle that provides mechanical stability for lower back pressure at higher flow rates. The amount of polyethylene glycol used in PEG-PS supports closely matches the hydrophilic nature of the growing peptide. As a result, they can be used for the synthesis of difficult peptides prone to form helices or sheets in the synthesis process.

DNA ProScan, **gel-reading software** that has been available for IBM and compat-

ible computers for over a year, is now available for the Macintosh from European Scientific Software (Reader Service No. 103). The system requires a MAC LC or higher, 4 MB of RAM, system 6.0 or higher, a colour monitor and a scanner capable of generating PICT files with 256 grey scales. The product is designed to take the errors out of reading gels. The system can be supplied with a 256 grey-scale, hand-held scanner for the Macintosh, which is used to generate gel images for analysis. European Scientific Software says that the software provides utilities that will enable you to read even difficult gels — gels with smiles, missing or faint bands. One feature of the software is in assisting the user to determine whether a particular band consists of 5 Gs or 4 Gs. Once read into the system, sequences can be stored in a variety of formats to allow further analysis by other analytical programs.

DNA sequences obtained from films and phosphor plates can now be read automatically using the new DNAscan software from Molecular Dynamics (*Reader Service No. 104*). DNAscan is an **automated DNA sequencing software program** and co-processor board compatible with the Molecular Dynamics computing densito-



DNA sequencing software.

meter, PhosphorImager and ImageQuant Workstation. The program automatically calls full-sized 35 × 43 cm DNA sequencing gels acquired by any of the company's scanners, generating the base sequence in seconds with little operator assistance. An automated lane finder follows lane centres of each of the lanesets on the gel, even when the lanes are compromised by 'curving' or 'smiling', the company says. Once the lanes have been identified, the individual bands in each lane are automatically located and the sequence is generated. DNAscan uses an algorithm that takes advantage of regional gel information as well as the 16-bit dynamic range of Molecular Dynamics' instruments to influence base assignments and increase the accuracy in the call. This, Molecular Dynamics says, translates to an increase in the number of calls and a reduced need for editing by the user. The i860-based co-processor board is suited to computer-intensive operations and allows DNAscan to run at speeds comparable to powerful workstations. The DNAscan graphical user interface includes a full complement of viewing and editing tools. In addition, the operator can annotate the file with other details specific to the experiment (such as source of DNA, vector, primer etcetera). DNAscan exports sequence and image data to spreadsheet and word processing programs in addition to multi-sequence analysis programs.

Clontech Laboratories announces new additions to its line of Quick-Clone cDNAs — high-purity, **double-stranded cDNA ready for use in the polymerase chain reaction (PCR)** (*Reader Service No. 105*). The newly available cDNAs include a line of plant cDNAs (for example, rice, tomato, corn, cotton, *Arabidopsis*, tobacco, potato, soybean and wheat). Others include human peripheral blood leukocyte, bone marrow and endothelial cell cDNAs and human fetal brain, kidney, liver and lung cDNAs. Over 60 Quick-Clone cDNAs are now avail-

able for several human, mouse, rat and plant tissues and for Chinese hamster ovary cells. Clontech says that the Quick-Clone cDNA offers researchers added convenience in isolating and cloning genes as it eliminates the need to purify RNA and synthesize cDNA. The cDNA is ready for PCR amplification; after purification, amplified cDNA regions can then be cloned, labelled for use as hybridization probes or directly sequenced. Quick-Clone cDNA is synthesized from poly A⁺ RNA using an oligo(dt)primer and then purified to remove interfering RNA and genomic DNA so that nonspecific amplification is reduced. In addition, Quick-Clone cDNA is size-selected to ensure a high probability of isolating full-length cDNAs. Each Quick-Clone cDNA includes two aliquots of cDNA, each of about 10 ng.

■ Probes

Oncor, a manufacturer of DNA probe-based systems for the early detection and management of cancer and other genetic diseases, has developed a **DNA probe for the detection of the *Her-2/neu* gene** directly on individual breast cells in tissue samples (*Reader Service No. 106*). "This development opens the door for the direct analysis of breast cells, to identify those lesions at high risk, using a simple, microscope slide-based procedure", said Stephen Turner, chairman and chief executive officer of Oncor. *Her-2/neu* is a cancer-causing gene that codes for a growth factor receptor protein. Oncor is supplying the product for research use.

Lagan has made available six new **genomic mapping probes**, which expands the company's LaganX.PLORER probe series to 28 (*Reader Service No. 107*). These probes provide for a quicker genomic mapping strategy through rapid contig assembly. LaganX.PLORER probes identify families of novel repetitive human DNA sequences called MER families, each of which is repeated 300 to 7,000 times per haploid genome. These families do not contain Alu, Kpn, O or THE repeats. Utilization of LaganX.PLORER probes using a two-step procedure is intended to provide for a faster contig assembly of human as well as higher primate DNAs cloned into YACs, cosmids and phages. In two hybridization steps, Lagan says that researchers can go from clones to blots to overlaps. LaganX.PLORER probes will not hybridize to

rodent DNAs, thereby making them useful tools in the analysis of rodent/human hybrid libraries.

The development of nonisotopic *in situ* hybridization techniques, such as FISH (fluorescence *in situ* hybridization) and biotin labelling, avoids the use of radioisotopes and can provide a quick answer to questions concerning ploidy, X-Y typing, specific chromosome centromere analysis and somatic cell hybrid analysis. Alpha Laboratories now offers a full range of **cytogenetic probes**, including all three major types of human satellite DNA — alpha, beta and the classical satellites (*Reader Service*



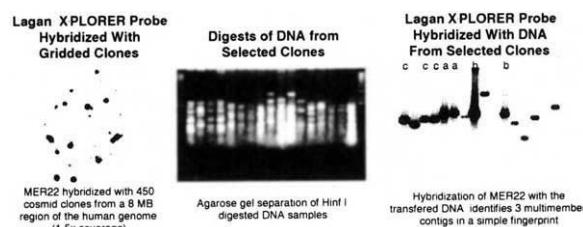
Cytogenetic probes for *in situ* hybridization.

No. 108). The range also includes an increasing number of whole chromosome paint probes. The probes are available separately or in kit form for fluorescence or light microscopy for fixed tissue, whole cells or metaphase spreads.

■ Kits

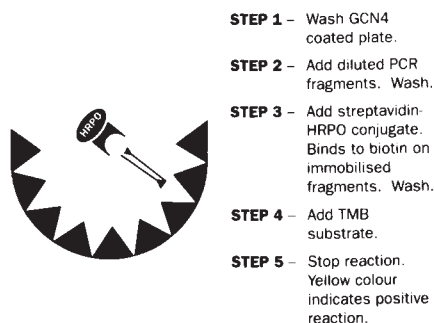
The pSTAMP plasmid amplification kit from International Laboratory Services follows hard on the heels of the company's pUCamp sister kit (*Reader Service No. 109*). Whereas pUCamp provides reagents to amplify plasmid inserts from within pUC-derived vectors, pSTAMP is targeted at a second group of vectors — those containing any two of the SP6/T3/T7 promoters in an opposed orientation. Taken together, the kits allow users to amplify insert DNA from clones in most commercially available plasmid vectors, independently of insert sequence. ILS says that the rapid and robust protocol (users can go from lysis to labelled probe in under four hours) can be used with DNA template from either purified plasmid or lysates of glycerol stock cultures. The amplification procedure in both kits can be used in a variety of applications, including the labelling of probes, DNA sequencing, site-directed mutagenesis, the characterization of clones and mapping experiments. The pSTAMP kit costs £79 (UK) and contains sufficient reagents for 20 reactions.

Captagene-GCN4 is British Bio-technology's new **enzyme-linked assay for the detection of amplified tar-**



From clones to blots to overlaps in two easy steps.

get DNA from the polymerase chain reaction (PCR) (*Reader Service No. 110*). The assay uses a microtitre plate-based format and therefore offers a convenient approach to the detection of PCR products when processing large sample numbers. It removes the need to run agarose gels and/or Southern blots for the detection of correctly amplified products. Hazardous reagents such as ethidium bromide or radioactive isotopes are not required. British Bio-technology says that the assay provides at least ten-fold greater sensitivity than agarose gels; ampli-



Captagene-GCN4 assay — see BBT.

fied DNA from 30 or more samples can be detected in 90 min. The GCN4 fusion protein (a highly specific, double-stranded DNA binding protein that recognizes the 12-base pair sequence 5'-GGATGACTCATT and which coats the microtitre plates in the Captagene-GCN4 assay) is also available separately as a pure reagent.

The isolation of DNA from lambda phage with acceptable purity can be difficult. The most commonly used procedure used to purify lambda DNA is a caesium chloride gradient, which can be cumbersome and which requires overnight ultracentrifugation. Biotex Laboratories has introduced two new kits, Lambda FaZe I and II, which are suitable for both minipreps and large-scale purifications and which, the company says, provide highly purified lambda DNA that is free from bacterial RNA, DNA, proteins and RNase (*Reader Service No. 111*). The resulting lambda DNA is suitable for restriction enzyme analysis, the polymerase chain reaction, *in vitro* transcription of RNA for its expression in *Xenopus* oocytes and *in vitro* translation, subcloning and sequencing.

Amersham has extended its range of nonradioactive detection products based on enhanced chemiluminescence (ECL) with the addition of its ECL probe-amp reagent (*Reader Service No. 112*). ECL probe-amp reagents are designed for the preparation of fluorescein-labelled DNA during the polymerase chain reaction (PCR) and for subsequent detection of these probes with ECL following hybridization. Amersham says that PCR-generated probes have several advantages over those obtained by more



Amersham's kit for the nonradioactive labelling of DNA probes during PCR.

traditional procedures: only small amounts of template are required, short probes can be labelled efficiently, no extensive purification of template is required and specific probes can be generated from heterogeneous sequences. In the ECL probe-amp reaction, fluorescein-11-dUTP is used as the label. Fluorescein-labelled PCR products can be examined using UV light following electrophoresis. The use of fluorescein as a hapten also offers the benefit that the degree of labelling can be measured in a rapid transilluminator-based assay, which makes use of the natural fluorescence of the hapten. Amersham says that the labelled probes are stable under standard hybridization conditions and can be used in a similar way to radioactively-labelled probes.

A new kit for site-directed mutagenesis from Boehringer Mannheim sets out to reduce the number of steps required to generate mutant clones (*Reader Service No. 113*). The kit uses uracil DNA glycosylase, which removes the nonmutagenized template DNA. Boehringer Mannheim says that the resulting clones can be screened directly by sequencing as mutation efficiencies of between 85 and 98 per cent are achieved. The time taken from production of template to isolation of clones is three days.

■ Manifolds

Immunetics introduces the Minislot 10, a vacuum manifold for use in the new checkerboard DNA hybridization or cross blot techniques (*Reader Service No. 114*). When used with the company's Miniblotter, Immunetics says that up to 280 separate hybridizations, antibody or other ligand-binding assays can be performed simultaneously on a single 7 × 10-cm membrane. In practice, solutions of DNA or antigens are



Minislot 10 vacuum manifold.

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Reader Service No.22

pipetted into each of the unit's ten slots and aspirated through a blotting membrane under vacuum, leaving horizontal stripes that span the width of the membrane. The membrane is then placed in a Miniblotter unit containing parallel, vertically arranged incubation channels. Different antibodies, DNA probes and other ligands introduced into each vertical channel are thus incubated with each of the ten horizontal stripes in a grid pattern. Enzymatic, chemiluminescent or radioactive methods can be used to detect binding reactions and the results can be scanned for quantitation. Immunetics points out that, compared with ELISA or dot blot techniques, the Minislot 10 unit and checkerboard procedure require smaller amounts of antigen and antibody and have fewer pipetting steps.

With the new GS Gene Prep **multiple-well filtration manifold** from Bio-Rad, users can simultaneously purify 24 single- or double-stranded DNA templates (using Bio-Rad's Prep-A-Gene matrix and an inert filter) (Reader Service No. 115). Bio-Rad



GS Gene Prep multiple-well manifold.

says that single-stranded DNA (M13 phagemid) can be purified for DNA sequence analysis directly from phage supernatants in as little as 45 min; double-stranded DNA (plasmid) can be purified for either DNA sequence analysis or restriction digestion from cleared bacterial lysates (alkaline lysis) in less than 2 h. The multiple-well format of the GS Gene Prep is designed to decrease the number of manipulations required to isolate large numbers of samples. The collection rack allows the orderly elution of samples from the manifold wells. Due to the selective DNA binding properties of the Prep-A-Gene matrix, proteins, RNA and other contaminants can be removed by washing with a chaotropic buffer, eliminating the need for phenol or chloroform extractions, Bio-Rad says.

Reagents

Calbiochem-Novabiochem now offers hygromycin B ($C_{20}H_{37}N_3O_{13}$, 350,000–500,000 U ml⁻¹ activity), an **antibiotic that inhibits the growth of microorganisms and mammalian cells**, as an aqueous solution (Reader Service No. 116). It has been shown that the gene (*hpr*) from *Escherichia*

coli that encodes resistance to hygromycin B can be isolated and cloned by recombinant DNA techniques. This gene is particularly useful for the identification or selection of recombinant clones in a variety of cell types. Calbiochem-Novabiochem says that using hygromycin B as an aqueous solution eliminates the need to handle toxic dusts and makes it easier to measure exact doses.

Pro-Cipitate, an insoluble polymeric reagent, is Affinity Technology's **alternative to phenol/chloroform** for the removal of contaminating proteins from nucleic acid preparations (Reader Service No. 117). The company says that Pro-Cipitate offers higher purity and yield of nucleic acids without exposure to toxic solvents and without residual reagents in the supernatant. The reagent can be incorporated into most protocols for the isolation of DNA and RNA and is suitable for both minipreps and large-scale isolations. Pro-Cipitate is supplied as a liquid suspension, ready for use and can be processed by centrifugation.

Catalogues/custom services

Life science researchers may be interested to hear that United States Biochemical's new 1993 Life Science Research Products **catalogue** has rolled off the presses (Reader Service No. 118). The catalogue, which complements the company's 1992 Molecular Biology Reagents/Products catalogue, is organized alphabetically to assist the user in finding what they are looking for among the more than 7,000 products. Also included are separate sections featuring the company's media for affinity chromatography, antisera, biological buffers, kits, laboratory animal test diets, lectins and restriction endonucleases.

Biomed's new 56-page **immuno-cytochemistry and molecular biology catalogue** contains technical information on kits and reagents for immunocytochemistry, immunology, molecular biology and cell biology (Reader Service No. 119). The catalogue also features fluorescent products for flow cytometry and a selection of mono- and polyclonal antibodies.

Multiple Peptide Systems now offers **five levels of peptide purity** to meet user requirements: crude, ≥55%, ≥80%, ≥95% and ≥98% (Reader Service No. 120). The company can also synthesize and purify peptides up to a purity of 99% by HPLC. All of the peptides are analysed by time-of-flight mass spectrometry for molecular identity and HPLC for purity. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.